Expression, Function, and Regulation of the K-Cl Cotransporter KCC2 isoforms in the Central Nervous System

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1 Table of Contents

ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
bp	base pair(s)
CCC	cation-chloride cotransporter
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CIP1	cation-chloride cotransporter - interacting protein 1
CNS	central nervous system
CoIP	co-immunoprecipitation
CREB	cAMP response element-binding protein
div	day *in vitro*
DNA	deoxyribonucleic acid
E	embryonic day
Egr	early growth response
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
HEK	human embryonic kidney
ICC	immunocytochemistry
IHC	immunohistochemistry
kb	kilobase pairs
KCC	K^+-Cl^- cotransporter
KO	knockout
LSO	lateral superior olivary nucleus
MAPK	mitogen-activated protein kinase
MHb	medial habenular nucleus
MNTB	medial nucleus of the trapezoid body
mRNA	messenger ribonucleic acid
MSO	medial superior olivary nucleus
N2a	neuroblastoma neuro-2a cell line
NCC	Na^+-Cl^- cotransporter
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
NMDAR	N-methyl-D-aspartate receptor
NRSE	neuron-restrictive silencing element
NRSF	neuron-restrictive silencing factor
OSR1	oxidative stress response-1
P	postnatal day
PCR	polymerase chain reaction
PFA	paraformaldehyde

PNS	peripheral nervous system
REST	restrictive element-1 silencing factor
RNA	ribonucleic acid
SLC	solute carrier
SOC	superior olivary complex
SPAK	Ste20-related proline-alanine-rich kinase
SPN	superior paraolivary nucleus
TSS	transcription start site
USF	upstream stimulatory factor
UTR	untranslated region
WB	Western blotting
WNK	with-no-lysine

ORIGINAL PUBLICATIONS/AUTHOR CONTRIBUTION

This thesis is based on the following publications, herein referred to by their Roman numerals (**I-III**):

I. Markkanen M*, Uvarov P*, Airaksinen MS. Role of upstream stimulating factors in the transcriptional regulation of the neuron-specific K-Cl cotransporter KCC2. Brain Res. 2008 Oct 21;1236: 8-15.
** Equal contribution. Markkanen M. participated in all experiments and in writing the manuscript.*

II. Markkanen M, Karhunen T, Llano O, Ludwig A, Rivera C, Uvarov P, Airaksinen MS. Distribution of neuronal KCC2a and KCC2b isoforms in mouse CNS. J. Comp. Neurol. 2014 Jun 1; 522(8): 1897-914.
Markkanen M. conducted immunostainings (Figs.1, 2C-E, 3, 4, 5, 6, 7 and 8) and participated in writing the manuscript.

III. Markkanen M, Ludwig A, Khirug S, Pryazhnikov E, Soni S, Khiroug L, Delpire E, Rivera C, Airaksinen MS, and Uvarov P. Implications of the N-Terminal Heterogeneity for the Neuronal K-Cl cotransporter KCC2 Function. Brain Res. 2017 Nov 15;1675: 87-101
Markkanen M. conducted several of the immunostaining experiments (Figs. 4a, 5, 7a and Supplemental Figs. 1,2 and 5), and participated in writing the manuscript.

Publication I is included in the doctoral thesis of PhD Pavel Uvarov (2010, University of Helsinki).

ABSTRACT

The potassium-chloride cotransporter KCC2 is a key regulator of chloride homeostasis in neurons of the central nervous system (CNS) and it is critical for the development of fast hyperpolarizing synaptic inhibition. Two full-length isoforms of KCC2, KCC2a and KCC2b, differing by their amino termini have been described. Both isoforms have similar expression levels in neonatal mice, but KCC2b is strongly upregulated in cortical areas during postnatal development, resulting in a developmental shift of GABAergic responses. In contrast to the well-studied KCC2b isoform, the importance of the KCC2a isoform has not yet been demonstrated. My thesis work focuses on characterizing the transcriptional regulation, expression and function of the KCC2 isoforms and a central aim is to elucidate the isoform-specific differences.

The molecular mechanisms underlying the regulation of KCC2 gene expression are not yet well understood. Both isoforms show a largely neuron-specific expression pattern and their expression is tightly regulated in development. The KCC2b isoform is also known to be regulated by both normal and pathological neuronal activity. One aim of the present thesis work was to explore the functionality of a conserved E-box site in the KCC2b promoter. Results suggest that the E-box site functions as a binding site for the upstream stimulating factors 1 and 2 (USF1, USF2), two basic helix-loop-helix transcription factors with potentially important roles in brain. Binding of USF proteins to the E-box motif contributes to the upregulation of KCC2b gene expression in immature cortical neurons.

Another aim of this study was to compare the postnatal expression patterns of KCC2a and KCC2b proteins in various regions of mouse CNS using immunohistochemistry and isoform-specific antibodies. The cellular expression patterns of KCC2a and KCC2b were largely similar in developing and neonatal mouse. In mature brain, KCC2a is detected in the basal forebrain, hypothalamus, and many areas of the brainstem and spinal cord, but its expression is very low in cortical regions. At the subcellular level, immunoreactivities of the isoforms are only partially colocalized, and KCC2a immunoreactivity, in contrast to KCC2b, is not clearly detected at the neuronal soma surface in most brain areas. Biotinylation experiments suggest that the N-terminal KCC2a epitope might be masked.

Results of this thesis work also indicate that the KCC2a isoform, similar to KCC2b, can function as a chloride transporter and decrease the intracellular chloride concentration in cultured neurons. The unique N-terminus of KCC2a includes a SPAK kinase binding site, and the importance of this site and the WNK-SPAK signaling pathway is also explored. Our results indicate that the SPAK kinase is able to bind the KCC2a isoform and to regulate the transport activity of KCC2a more than that of KCC2b.

1. REVIEW OF THE LITERATURE

1.1 THE CCC FAMILY AND KCC2

The electroneutral cation-chloride cotransporters (CCCs) are integral plasma membrane proteins that belong to the solute carrier 12 (*SLC12*) gene family. CCCs mediate the coupled transport of Cl^- and cations (K^+ and/or Na^+) across the plasma membrane. The gradients of Na^+ and K^+ are established by the Na^+-K^+-ATPase and provide the energy for the transport of Cl^-. Under normal physiological conditions the Na^+ driven cotransporters transport chloride into the cell, while chloride efflux is powered by the K^+ driven cotransporters. However, CCCs are bidirectional and can mediate a net efflux or influx, depending on existing ionic gradients (Gamba, 2005, Kaila *et al*, 2014).

In mammals the CCC family has nine members: Na^+ driven CCC members include two Na^+ -K^+ -2 Cl^- cotransporters (NKCC1-2), and one Na^+ - Cl^- cotransporter (NCC), while K^+ driven CCCs include four K^+- Cl^- cotransporters (KCC1-4). CCC9 and the CCC interacting protein 1(CIP1) are also members of the CCC family but their physiological role is not known (Gagnon and Delpire, 2013).

Significant isoform diversity within the CCC family is generated by alternative splicing events, and the isoforms often demonstrate differences in tissue distribution or functional characteristics (Gamba, 2005). Expression and function of CCCs is regulated at the transcriptional and post-translational levels. Phosphorylation-dephosphorylation, in particular, is a key mechanism of regulation of CCCs at the protein level (Medina *et al*, 2014).

Important functional roles of CCCs include intracellular Cl^- homeostasis and cell volume regulation, as the transported ions are also accompanied by water (Kahle *et al*, 2015). Some members also have specific roles in epithelia and neurons. Of the N^+ driven cotransporters, NKCC1 is widely expressed and has various functions in Cl^- homeostasis, cell volume regulation and epithelial transport. NKCC2 and NCC are involved in renal salt reabsorption in the kidney, but expression NKCC2 has also been observed in the hypothalamo-neurohypophyseal system in rat brain (Konopacka *et al*, 2015). Of the K^+ driven cotransporters, KCC1 is ubiquitously expressed while KCC3 and KCC4 have a somewhat more restricted expression (Gillen *et al*, 1996, Mount *et al*, 1999). KCC3 is expressed in various tissues, including brain, and it is particularly important for cell volume regulation following hyposmotic swelling (Rust *et al*, 2006, Boettger *et al*, 2003, Byun and Delpire, 2007). KCC4 is known to function in transepithelial transport in the inner ear and kidney (Boettger *et al*, 2002).

Among the KCCs, KCC2 is unique as it is central nervous system (CNS) neuron specific and has important functions in neurons, especially as it mediates an efficient Cl- extrusion in mature neurons (Payne *et al*, 1996, Rivera *et al*, 1999, Hubner *et al*, 2001). The intracellular Cl^- concentration of neurons determines the strength and polarity of transmission via type A γ-

aminobutyric acid (GABA) receptors and glycine receptors, which are both Cl^--permeable ion channels. KCC2 also appears to interact with components of the cytoskeleton in neurons, and to play an important morphogenic role in dendritic spine formation and function of excitatory synapses in the CNS (Li *et al*, 2007, Fiumelli *et al*, 2013, Horn *et al*, 2010, Llano *et al*, 2015, Gauvain *et al*, 2011).

The KCC2 gene generates two splice variants, KCC2a and KCC2b, by two alternate first exons under control of separate promoters (Uvarov *et al*, 2007). KCC2a and KCC2b isoforms differ only in the amino (N)-terminus encoded by these two alternate first exons. In the neonatal mouse brain, the two KCC2 isoforms have similar protein levels and distribution, are co-expressed in many neurons (Uvarov *et al*, 2009). The KCC2a protein shows only moderate increase during postnatal development whereas KCC2b is strongly upregulated in development. In adult mouse brain, KCC2b is the predominant isoform and KCC2a makes up less than 10% of the total KCC2 (Uvarov *et al*, 2007, Uvarov *et al*, 2009).

Members of the CCC family are associated with various diseases, and KCC2 in particular has been associated with neurological or neuropsychiatric diseases. Downregulation of KCC2 expression or activity is associated with elevated levels of intracellular chloride, resulting in enhanced neuronal activity, hyperexcitability and seizures. Epileptiform activity has been observed in studies in animal models (Rivera *et al*, 2002, Rivera *et al*, 2004) and humans (Huberfeld *et al*, 2007). Mutations in KCC2 have also been shown to be associated with epilepsy in humans (Kahle *et al*, 2014, Puskarjov *et al*, 2014, Stodberg *et al*, 2015, Saitsu *et al*, 2016). Downregulation of KCC2 is also observed for example in neuropathic pain (Coull *et al*, 2003), brain trauma (Shulga *et al*, 2008) and spasticity after spinal cord injury (Boulenguez *et al*, 2010).

Moreover, changes in KCC2 gene transcription from early stages may contribute to the genetic risk of neurodevelopmental disorders in humans. Dysregulation of Cl^- homeostasis and abnormalities in GABA signaling during development may disrupt the trophic effects of GABA and affect a variety of developmental processes such as migration, differentiation, synapse maturation, and neuronal wiring (Cellot and Cherubini, 2014). Changed expression of chloride cotransporters has been implicated in autism spectrum disorders such as Rett syndrome (Tang *et al*, 2016) and Fragile X syndrome (He *et al*, 2014, Tyzio *et al*, 2014) and schizophrenia (Hyde *et al*, 2011, Tao *et al*, 2012) .

1.2 THE KCC2 GENE

1.2.1 Gene structure

The CCC family is highly conserved in evolution, and homologs of CCCs are present in various eukaryotes (Gagnon and Delpire, 2013). Prokaryotic homologs of the CCC family have also been identified. Many species of vertebrates have four genes encoding K^+-driven transporters (KCC1-4; genes *Slc12a4-7*) as well as three Na+ -driven transporters (NKCC1

and 2 and NCC; genes *Slc12a1-3*). During vertebrate evolution, the ancestral KCC coding gene probably gave rise to KCC1 and KCC3 in one duplication and KCC2 and KCC4 in another duplication, resulting in the four KCCs (Gagnon and Delpire, 2013, Hartmann *et al*, 2014).

In *Drosophila melanogaster* there are five CCC genes including one *kcc*-like gene (kazachoc) (Rusan *et al*, 2014). The *kcc* gene of drosophila is important for inhibitory neurotransmission (Hekmat-Scafe *et al*, 2006), suggesting that the K^+ -driven transporters initially evolved to serve this particular function in the nervous system. The *Caenorhabditis elegans* genome contains seven CCC isoforms including three *KCC* genes, and the function of one (*ce-KCC2*) is important for inhibitory neurotransmission and synapse maturation (Tanis *et al*, 2009).

The mammalian KCC2 gene (*Slc12a5*) consists of 27 exons and is located on chromosome 2 in mouse (Fig. 1A,B). The gene has two alternative first exons (1a and 1b) and two transcripts with 26 exons are generated, each encoding a full-length protein isoform with a predicted topology of twelve transmembrane spanning segments and intracellular N- and C –termini (Uvarov *et al*, 2007, Payne *et al*, 1996) (Fig. 1C). KCC2a transcripts lacking exon 24 are expressed in pancreatic β-cells and in adrenal medullary cells (Kursan *et al*, 2017) (Fig. 1C).

The coding part of the first exon of KCC2a in mouse contains 121 base pairs (bp) (encodes 40 amino acids), while the coding part of the first exon of KCC2b contains 52 bp (encodes 17 amino acids) (Fig. 2).

The exon structure of the *KCC* genes is quite similar in mammals, but exons 22 and 24 of KCC2 (encoding portions of the C-terminal domain), are absent from *KCC* genes in most mammals (Payne *et al*, 1996). Exon 22 of KCC2 (encodes 41 amino acids), is absent from *KCC1* and KCC3 genes of vertebrates, but it is present in *KCC4* of lower vertebrates, including fish, birds, and a prototherian mammal (platypus). Exon 24 of KCC2 (encodes 5 amino acids) is also missing in most *KCCs*, but it has been identified in some alternatively spliced variants of the KCC4 gene (*Slc12a7*) in mammals (Antrobus *et al*, 2012).

Several truncated transcripts have been described for KCC2b in patients with schizophrenia (Tao *et al*, 2012), but the significance of these transcripts is not known, as the encoded proteins would most likely be non-functional in ion transport.

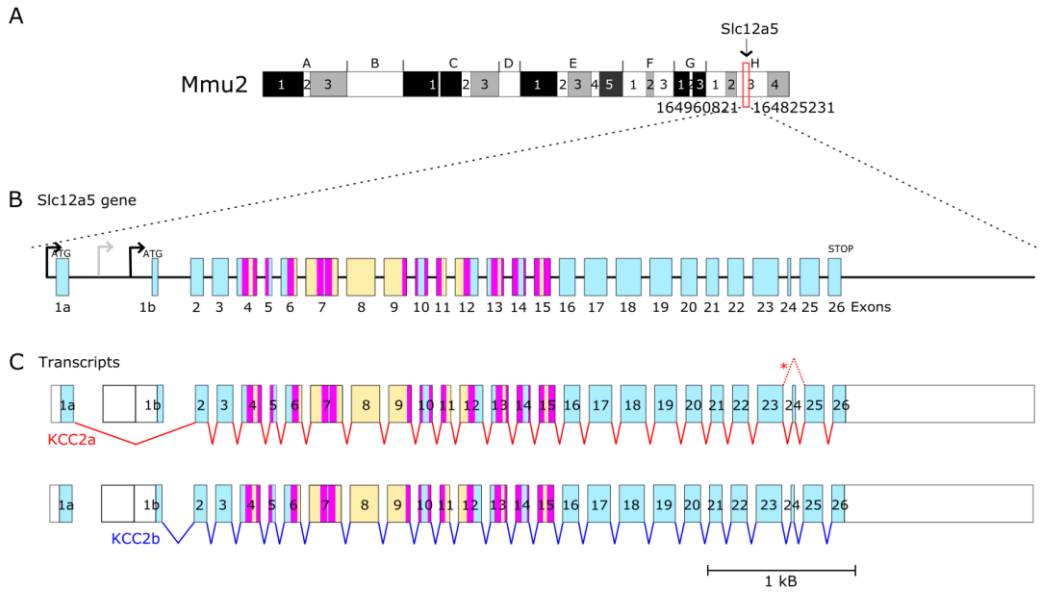


Fig.1 Chromosome location, gene structure and full-length transcripts of *Slc12a5*
A) The *Slc12a5* gene is located on chromosome 2 in mouse. The nucleotide base pairs indicate the start and stop of the gene according to the assembly GRCm38 for mouse.
B) Exon organization of the *Slc12a5* gene: exons are illustrated as boxes, introns as lines (the latter are not drawn to scale). The full-length protein isoforms have a predicted topology of twelve transmembrane spanning segments and intracellular N- and C –termini and these different domains are depicted here as colored regions of the coding exons: transmembrane domains (pink), intracellular parts (blue), extracellular parts (yellow). Main transcription start sites are shown by black arrows, and a minor transcription start site is represented by a gray arrow (Payne *et al*, 1996, Uvarov *et al*, 2005).
C) Full-length KCC2a and KCC2b mRNA transcripts, consisting of 26 exons each, with alternatively spliced first exons are generated. Untranslated regions (UTRs) of transcripts are represented by white boxes. Alternative splicing of exon24 (marked by an asterisks) generates a KCC2a variant identified in pancreas.

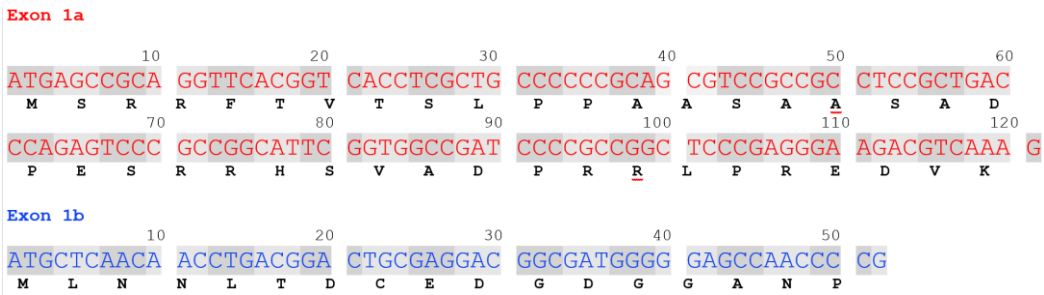


Fig. 2 Coding parts of exon 1a and exon 1b
Sequences of the coding parts of exon 1a (121 bp) and exon 1b (52 bp), as well as encoded amino acids, are shown.

1.2.2 mRNA expression pattern

In situ hybridization using probes detecting both KCC2 isoforms show that KCC2 mRNA expression is strongly upregulated during development and the upregulation is correlated with the maturation of neurons in rodents (Li *et al*, 2002, Wang *et al*, 2002, Stein *et al*, 2004). KCC2 mRNA is observed in differentiated neurons but not in neuronal precursors or in migrating cells (Li *et al*, 2002, Wang *et al*, 2002, Stein *et al*, 2004). The first KCC2 transcripts are detected in mice in the ventral horn of the spinal cord and in the immature brainstem by embryonic day 10.5 (E10.5) (Stein *et al*, 2004). Gradually the expression spreads in a caudal-rostral fashion: At E14.5, expression is found in the diencephalon, in hypothalamus, ventral thalamus and ventral lateral geniculate nucleus and moderate levels are observed in the dorsal parts of thalamus. In telencephalon, expression can be seen in the olfactory bulb at this time-point (Li *et al*, 2002, Wang *et al*, 2002, Stein *et al*, 2004).

At postnatal day 0 (P0), KCC2 mRNA expression has spread throughout diencephalon, and a few regions of telencephalon show expression: namely basal ganglia, piriform cortex, amygdala and olfactory bulb (Li *et al*, 2002, Wang *et al*, 2002, Ikeda *et al*, 2003). During further postnatal development, expression gradually spreads into higher brain structures such as neocortex, hippocampus and cerebellum. The adult level and pattern of KCC2 mRNA expression, including strong expression in neocortex and hippocampus, is reached at P15 in mice (Stein *et al*, 2004, Wang *et al*, 2002, Rivera *et al*, 1999). The KCC2 mRNA expression follows a similar pattern in mouse and rat, but in rat expression is delayed by approximately two days (Li *et al*, 2002).

KCC2 transcripts are expressed in mature neurons throughout the CNS but not in PNS (except for some regions, see below). Some CNS areas where KCC2 mRNA has not been detected are the mesencephalic trigeminal nucleus (Kanaka *et al*, 2001, Toyoda *et al*, 2005), vasopressin-positive neurons in the thalamus (Bartho *et al*, 2004, Kanaka *et al*, 2001), dopaminergic neurons in substantia nigra pars compacta (Gulacsi *et al*, 2003), dorsolateral part of the paraventricular nucleus (Kanaka *et al*, 2001), dorsomedial part of the suprachiasmatic nucleus (Kanaka *et al*, 2001), ventromedial part of the supraoptic nucleus (Kanaka *et al*, 2001, Leupen *et al*, 2003), reticular thalamic nucleus (Kanaka *et al*, 2001, Bartho *et al*, 2004) and medial habenular nucleus (Kanaka *et al*, 2001).

Only the KCC2b isoform undergoes strong up-regulation during postnatal development, whereas KCC2a mRNA expression remains relatively constant (Uvarov *et al*, 2007). In quantitative real-time PCR, the KCC2a and KCC2b mRNA expression was detected in the brainstem, spinal cord, and olfactory bulb at E17, and the relative expression of the isoforms was similar in these brain regions. KCC2a and KCC2b mRNA signals were very low in cortex and hippocampus at E17. Between E17 and P14, relative KCC2b mRNA levels increased strongly in the neocortex (by 35-fold) and hippocampus (by 10-fold), while relative KCC2a mRNA levels did not change significantly (increased only ~2-fold in cortex, and decreased slightly in hippocampus, spinal cord and brain stem). In adult mouse brain the level of KCC2a mRNA consisted of only 4–8% of total KCC2 mRNA, as measured by RNA protection assay (Uvarov *et al*, 2007).

The expression pattern of KCC2 isoforms is largely CNS neuron specific (Payne *et al*, 1996, Uvarov *et al*, 2007). However, KCC2 transcripts have been detected in cultured rat vascular smooth muscle cells (Di Fulvio *et al*, 2001) and several human cancer cell lines (Wei *et al*, 2011). KCC2a transcripts have also been detected in human lens epithelial cells (Lauf *et al*, 2012), in chicken cardiomyocytes (Antrobus *et al*, 2012) and in pancreatic beta cells (Kursan *et al*, 2017).

1.2.3 Regulation of gene expression

1.2.3.1 KCC2 promoter regions

The main transcription start sites and the core promoter region of KCC2b gene have been identified (Payne *et al*, 1996, Uvarov *et al*, 2005) (Fig 3). No typical TATA or CAAT boxes are present in the KCC2 promoter region. In transiently transfected mouse neuroblastoma N2a cells, an approximately 300bp sequence region upstream of the main TSS was sufficient to provide a basal level of KCC2 promoter activity, and most of the basal activity was defined by the sequence −180 to +42 around the TSS region (Uvarov *et al*, 2005, Uvarov *et al*, 2006, Uvarov *et al*, 2007). The activity of a KCC2 promoter construct containing intron 1b sequence was inhibited in N2a cells, and thus a downstream response element within the intron 1b region presumably functions as a transcriptional silencer (Uvarov *et al*, 2005). The KCC2a promoter region (approximately 1 kb of mouse genomic sequence upstream of the KCC2a TSS) also possesses transcription activity in cultured neurons (Uvarov *et al*, 2007).

Several consensus sequences of transcription factor binding sites conserved in mammals have been identified in the KCC2b promoter and proximal intron-1 regions (Uvarov *et al*, 2006) (Fig 3). The -180/+42 region contains only the TSS and Sp1 and AP2 consensus sequences. Early growth response 4 (Egr4) binds to an Egr-responsive element in the KCC2b promoter (Uvarov *et al*, 2006). Two conserved neuron-restrictive silencing elements (NRSEs) (also called RE1 motif) associated with the KCC2 gene have been shown to bind neuron-restrictive silencer factor NRSF (also called repressor element-1 silencing transcription factor, REST) (Karadsheh and Delpire, 2001, Uvarov *et al*, 2005, Yeo *et al*, 2009). The predicted proximal KCC2a promoter contains a putative TATA box and conserved binding sites for ubiquitous transcription factors such as E2F and HNF4 but no obvious conserved sites for neuron-enriched transcription factors (Uvarov *et al*, 2007).

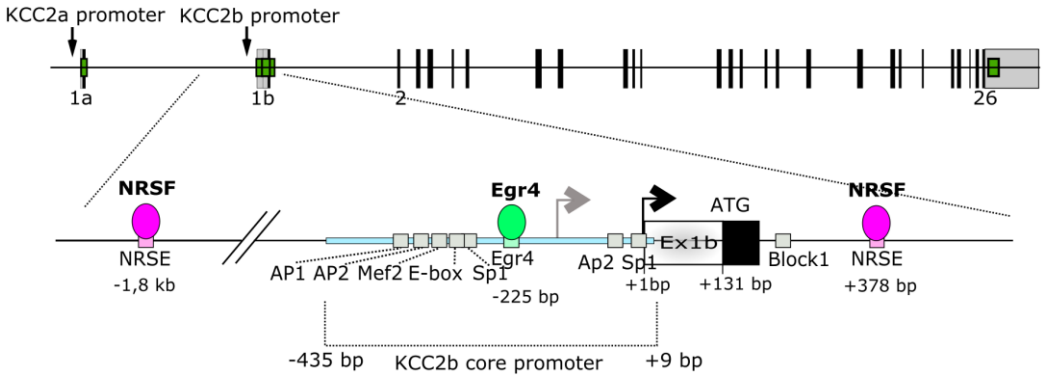


Fig. 3. KCC2b promoter region
Schematic representation of the KCC2 genomic region with isoform specific promoters indicated. Five putative CpG islands (shown in green) are predicted within the KCC2 genomic region: one in the promoter/5′-UTR region of exon1a, three in the KCC2b promoter/5′-UTR region, and one in the 3′-UTR region of the KCC2 gene (Uvarov *et al*, 2005, Uvarov *et al*, 2007). The upstream promoter region of exon 1b and part of intron 1b are shown in detail. Positions of TSSs are indicated by curved arrows: The main TSS, where 90-95% of transcripts are started, is designated by +1 (black curved arrow). Several minor transcription initiation sites are present further upstream, the most upstream is indicated (gray curved arrow). The translation initiation codon (ATG) is indicated. The region showed in blue, located −435 to +9 around the TSS, is predicted to be the core promoter of the KCC2 gene and has a GC content above 70%. Potential binding sites for transcription factors are shown: neuron-restrictive silencer element (NRSE), activating enhancer-binding protein 1 (AP1) and 2 (AP2), myocyte-enhancing factor 2 (Mef2), E-box, specificity protein 1 (Sp1), early growth response 4 (Egr4), block1. Binding of transcription factors Egr4 and neuron-restrictive silencer factor (NRSF), which are both implicated in KCC2b gene regulation, are shown.

1.2.3.2 Mechanisms of KCC2 gene regulation

KCC2 mRNA expression follows neuronal maturation and synaptogenesis (Li *et al*, 2002, Gulyas *et al*, 2001, Rivera *et al*, 2005). Suggested mechanisms underlying the KCC2 upregulation in development include depolarizing responses to GABA in immature neurons and the resulting influx of Ca^{2+} via voltage-gated calcium channels (Ganguly *et al*, 2001). However, it has been shown that neither GABA type A receptor activation, extrasynaptically released GABA, synaptic transmission or action potentials were necessary for the expression of KCC2 in development (Ludwig *et al*, 2003, Titz *et al*, 2003, Wojcik *et al*, 2006). Therefore, other factors related to the maturation and synapse-formation might be important, such as cell to cell interactions and the release of growth factors (Ludwig *et al*, 2003, Kelsch *et al*, 2001, Aguado *et al*, 2003).

Brain derived neurotrophic factor (BDNF) favors KCC2 mRNA expression in immature hippocampal neurons (Ludwig, Uvarov, Soni *et al*, 2011). BDNF binds to the tropomyosin receptor kinase B (TrkB) receptor and activates intracellular cascades that results in the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway and the mitogen-activated protein kinase (MAPK) pathway. Activation of ERK1/2 induces enhanced Egr4 expression and Egr4-dependent increase of KCC2 (Ludwig *et al*, 2011). The trophic factor neurturin also increases KCC2 expression in developing neurons via the ERK1/2 pathway, triggering Egr4 mRNA expression and upregulates the KCC2 protein (Ludwig, Uvarov, Pellegrino *et al*, 2011).

A decrease in KCC2 mRNA has been observed in several pathological conditions for example in epilepsy (Palma *et al*, 2006, Huberfeld *et al*, 2007), chronic pain (Coull *et al*, 2003), spasticity after spinal cord injury (Boulenguez *et al*, 2010), motor neurons after axonal injury (Nabekura *et al*, 2002, Toyoda *et al*, 2003, Shulga *et al*, 2008, Shulga *et al*, 2009), and in spinal cord neurons after sciatic nerve lesion (Coull *et al*, 2003). Activity-dependent regulation of KCC2 mRNA has been observed in hippocampal neurons after kindling-induced seizures and exogenous BDNF application (Rivera *et al*, 2002). In mature hippocampal neurons, sustained interictal-like activity induced BDNF release and the BDNF-TrkB signaling resulted in a down regulation of KCC2 gene expression via the transcription factor cAMP response element-binding protein (CREB) (Rivera *et al*, 2004).

KCC2b mRNA is specifically expressed in neurons of the CNS, and one mechanism that may contribute to the neuron-specific expression of KCC2b is the NRSF binding to NRSE sites in the KCC2 gene. The NRSF functions to suppress transcription of NRSE containing genes in non-neuronal tissues by assembling a multiprotein complex to modify covalent modification of chromatin (Schoenherr and Anderson, 1995, Naruse *et al*, 1999, Roopra *et al*, 2000). The NRSE in the KCC2 intron-1b has been demonstrated to interact with the NRSF and suppress the transcriptional activity of the KCC2 1b promoter *in vitro* (Karadsheh and Delpire, 2001). Another NRSE in KCC2 intron 1a (1.8 kb upstream of the KCC2b TSS) also mediated transcriptional inhibition of the KCC2b *in vitro* ((Karadsheh and Delpire, 2001, Uvarov *et al*, 2005, Yeo *et al*, 2009). However, NRSF binding seems not to be essential for the neuron-specific expression pattern of KCC2 since a KCC2 transgene containing a 1.4kb genomic fragment containing neither of the two NRSE sites was still expressed predominantly in CNS neurons in transgenic mice (Uvarov *et al*, 2005).

Epigenetic factors including DNA methylation and proteins binding methylated DNA might be important in controlling the expression of the KCC2 gene. The methyl-CpG binding protein 2 (MeCP2) has been implicated in KCC2 gene regulation via inhibiting NRSF binding to the NRSE sites and thus preventing NRSF mediated inhibition of KCC2 expression (Tang *et al*, 2016). In Rett neurons, where MeCP2 is deficient, NRSF can bind to the NRSE sites and suppress KCC2 expression (Yeo *et al*, 2009). DNA methylation and MeCP2 binding was observed to exert a repressive effect on 2.5 kb of the KCC2 promoter surrounding the TSS (Yeo *et al*, 2013). Several CG dinucleotides that are methylated in non-neural tissues but

unmethylated in neurons were identified in the KCC2b promoter and methylation might be important in the neuron-specific expression of KCC2.

The 3'-UTR region of the KCC2 gene is long and contains a CpG island (Uvarov *et al*, 2007). A putative promoter situated in the 3'-UTR region was suggested to drive an antisense RNA that can inactivate the KCC2 gene (Akan *et al*, 2009). The KCC2 3'-UTR region also contains several conserved target sites for microRNA-92 which was found to interact with KCC2 mRNA and reduce its translation (Barbato *et al*, 2010). Training mice in contextual fear conditioning produced a transient increase in microRNA-92 levels in the hippocampus that lead to the downregulation of KCC2 protein (Vetere *et al*, 2014).

1.3 KCC2 PROTEIN ISOFORMS

1.3.1 Structure and interactions

The full-length KCC2 transcripts in mouse encode proteins with 1138 (KCC2a) and 1115 (KCC2b) amino acids, and predicted molecular mass of ~125 kDa (Uvarov *et al*, 2007). The predicted topology of the full-length plasmalemmal CCC family members consists of 12 alpha-helical transmembrane segments flanked by intracellular amino (N) and carboxy (C) terminals (Payne *et al*, 1996) (Fig. 4). A large glycosylated extracellular loop is present between transmembrane domains 5 and 6 in KCCs, and the glycosylated molecular mass of KCC2 is ~140 kDa (Payne *et al*, 1996). Six glycosylation sites have been identified by mass spectrometry (Agez *et al*, 2017) (Fig. 4).

The long intracellular C-terminal domain is highly conserved among CCCs. The C-terminal domain of KCC2 seems to be important for surface stabilization, as truncation of the C-terminus resulted in rapid internalization (Friedel *et al*, 2017).The C-terminal domain also influences the cotransport function of KCC2 (Casula *et al*, 2001, Shen *et al*, 2003, Li *et al*, 2007, Horn *et al*, 2010, Fiumelli *et al*, 2013, Strange *et al*, 2000). KCC2 is the only KCC that exhibits activity under isotonic conditions while the three other KCCs need to be activated by hypotonic conditions. The isotonic activity of KCC2 is provided by a unique ISO-domain in the C-terminal part of the transporter, encoded by residues in exon 23 (Mercado *et al*, 2006, Acton *et al*, 2012).

The KCC2 protein contains several sites important for functional regulation and protein-protein interactions (Fig. 4). Cysteine residues in the large extracellular loop of KCC2 (C287, C302, C322, C331) (Hartmann *et al*, 2010), as well as a cysteine residue C568 in the 10th putative transmembrane domain (Reynolds *et al*, 2008) are important for the ion transport activity of KCC2. C568 is possibly also important for structural interactions of KCC2 with the cytoskeleton (Horn *et al*, 2010). Leucine 675 (L675) is also involved in KCC2 ion-transport activity (Doding *et al*, 2012). Several missense mutations in KCC2 in humans have been associated with epilepsy: R952H and R1049C (Kahle *et al*, 2014, Puskarjov *et al*, 2014),

L426P, G551D and L311H (L403P, G528D, and L288H in KCC2b) (Stodberg *et al*, 2015), M415V A191V S323P, as well as a deletion in the N-terminus (E50-Q93) by skipping of exon 3 (Saitsu *et al*, 2016). The mutation W318S and a deletion of S748 in the C terminus were also identified, but the impact of these mutations on KCC2 was not examined. Mutations R952H and R1049C as well as R1048W in human KCC2 were associated with autism (Merner *et al*, 2015).

Several phosphorylated residues have been identified in KCC2: serines 728 and 940 (S728; S940) (Lee *et al*, 2010, Lee *et al*, 2007), threonines 906 and 1007 (T906, T1007) (Rinehart *et al*, 2009) and tyrosines 903 and 1087 (Y903 and Y1087) (Lee *et al*, 2010). In addition, several predicted sites of KCC2 phosphorylation have been identified (Payne *et al*, 1996, Weber *et al*, 2014) (Fig. 4).

The last 18 C-terminal amino acids in all CCCs are important for the interaction with brain-type creatine kinase (CKB) (Inoue *et al*, 2004). KCC2 also interacts with the α2 subunit of the Na-K ATPase (K. Ikeda *et al*, 2004) and with the protein associated with Myc (PAM) (Garbarini and Delpire, 2008). KCC2 C-terminus contains a non-canonical di-leucine motif that interacts with the clathrin-binding adaptator protein-2 (AP-2) (Zhao *et al*, 2008). Moreover, KCC2 interacts with several synaptic proteins: GABAA receptors (Huang *et al*, 2012), the GluK2 subunit of kainate receptors (Mahadevan *et al*, 2014) and its interacting protein Neto2 (Ivakine *et al*, 2013). KCC2 through its C-terminal domain also interacts with actin related proteins 4.1N (Li *et al*, 2007) and Beta-PIX (Llano *et al*, 2015).

The N-terminal part of KCCs is highly variable and often subject to alternative splicing, as in KCC2 (Uvarov *et al*, 2007). Truncation of the N-terminus of KCC2 has been shown to inhibit the ion-transport function (Li *et al*, 2007) and surface expression (Friedel *et al*, 2017). KCC2a and KCC2b, however have similar ion-transport properties when expressed in human embryonic kidney (HEK) cells (Uvarov *et al*, 2007). The SPAK binding sequence RFx(V/I) is present in N-terminal domains of several CCC family isoforms (Delpire and Gagnon, 2008, Richardson *et al*, 2008) and SPAK has been identified as a binding partner of all CCCs (Piechotta *et al*, 2002). In KCC2, the a-isoform specific N-terminal part contains a SPAK binding sequence and SPAK was also shown to interact with KCC2a (Uvarov *et al*, 2009). Several residues have been shown to be directly phosphorylated by the SPAK kinase: T6 (a-isoform specific) T77, S78, S83, T92, and T1007 (de Los Heros *et al*, 2014).

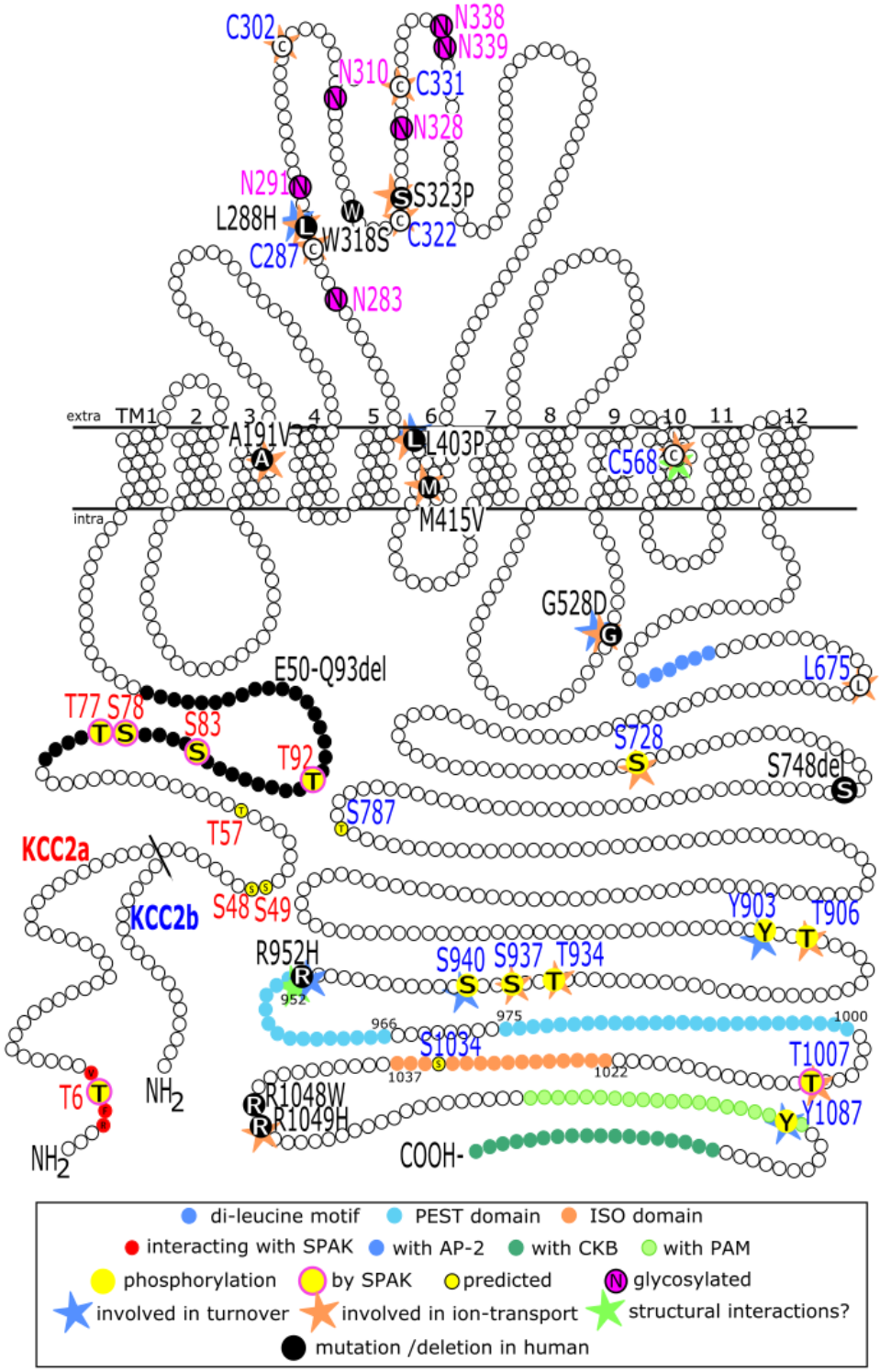
CCCs are suggested to exist as functional homo- or heterodimers in the plasma membrane (Medina *et al*, 2014). KCC2 proteins are also demonstrated to exist as functional oligomers in the plasma membrane (Blaesse *et al*, 2006) and KCC2a and KCC2b may also form heteromers *in vivo* (Uvarov *et al*, 2009). However, it is not clear how oligomerization affects ion-transport activity, or what the functional relevance of heterodimers is. Recently KCC2 was found to exist as monomers and dimers in the plasma membrane when overexpressed in HEK293 cells, and no higher oligomers or aggregates were observed in Native PAGE experiments (Agez *et al*, 2017). Moreover, both the KCC2 monomers and dimers were functional. Based on electron microscopy analysis, a 3-D model was suggested where asymmetrical KCC2

dimers are formed via functional homodimerization of the C-terminal domain while disulfide bridges also are important in dimerization (Agez *et al*, 2017).

KCC2 in the plasma membrane is internalized and recycled back to the plasma membrane through constitutive endocytosis mediated by dynamin and clathrin in HEK293 cells (Zhao *et al*, 2008). In addition, PACSIN1 was found to exist in a complex with KCC2 in mouse brain (Mahadevan *et al*, 2017). PACSIN1 is an endocytic adapter protein that plays important roles in postsynaptic receptor recycling. A half-time turnover rate close to 10 min was estimated for KCC2 under basal conditions (Rivera *et al*, 2004, Lee *et al*, 2007, Zhao *et al*, 2008, Lee *et al*, 2010) but was estimated to be several hours or days in another study using brain slices (Puskarjov *et al*, 2012). The C-terminal domain also contains two predicted PEST (Proline/E (glutamate)/Serine/Threonine) sequences that are unique to KCC2 and suggested to function in targeting proteins for rapid degradation (Mercado *et al*, 2006).

The expression and function of KCC2 at the membrane is regulated by multiple pathways via various post-translational modifications. Rapid internalization of KCC2 is seen following an increase in neuronal or synaptic activity (Chamma *et al*, 2013, Fiumelli *et al*, 2005, Watanabe *et al*, 2009, Lee *et al*, 2011, Kahle *et al*, 2013). Activity-induced dephosphorylation of Ser940 and phosphorylation of Tyr903/1087 leads to increased endocytosis of KCC2 (Lee *et al*, 2010, Lee *et al*, 2011). Ca^{2+} influx through N-methyl-D-aspartate receptors (NMDARs) leads to a calcium dependent Ser940 dephosphorylation and resulted in calpain-mediated cleavage of the C-terminal domain and KCC2 internalization (Puskarjov *et al*, 2012, Chamma *et al*, 2013). The interaction between KCC2 and the endocytic regulatory protein PACSIN1 restricts the expression and activity of KCC2 in hippocampal neurons (Mahadevan *et al*, 2017).

Fig. 4 Topology model of the KCC2 protein
The model represents the mammalian KCC2 protein, and N-terminal parts of both isoforms (KCC2a and KCC2b) are shown. Functional domains as well as domains for protein-protein interactions are indicated. Phosphorylated residues and predicted phosphorylation sites, as well as residues involved in turnover (surface stability and/or internalization mechanism), ion-transport function and possible structural interactions are also shown. Numbering is according to KCC2a in the N-terminal part (in red), otherwise numbering corresponds to the KCC2b sequence.


C302
N338
N339
N310
C331
N328
N291
S323P
L288H
W318S
C322
C287
N283
extra
TM1
2
3
4
5
6
7
8
9
10
11
12
intra
A191V
L403P
C568
M415V
G528D
E50-Q93del
L675
T77 S78
S83
T92
S728
S748del
S787
T57
KCC2a
KCC2b
S48 S49
Y903
T906
R952H
S940 S937 T934
952
966
975
1000
S1034
1037
1022
T1007
T6
NH2
R1048W
R1049H
Y1087
NH2
COOH-
di-leucine motif
PEST domain
ISO domain
interacting with SPAK
with AP-2
with CKB
with PAM
phosphorylation
by SPAK
predicted
glycosylated
involved in turnover
involved in ion-transport
structural interactions?
mutation /deletion in human

1.3.2 Protein expression

In Western blot analysis with antibody against the KCC2b isoform, the KCC2b protein expression in mouse brain is characterized by an increase during development, similar to what is observed at the mRNA level (Stein *et al*, 2004). In mouse spinal cord and brainstem, KCC2b signals were intense already at E15.5 and did not further increase beyond P3. In mouse cerebellum and cortex KCC2b was first detectable at E15.5 and in hippocampus at P3. A strong KCC2b protein upregulation was observed in cerebellum, hippocampus, and cortex between E15.5 and P15 (Stein *et al*, 2004).

Western blot analysis with antibody against the KCC2a isoform has demonstrated that KCC2a expression changes only moderately during postnatal mouse development (Uvarov *et al*, 2009). KCC2a protein levels between P2 and adult time-points were about 2-fold decreased in brainstem and spinal cord and 2-fold higher in cortex. Total KCC2 (KCC2pan antibody recognizing both isoforms) protein levels between P2 and adult time-points was not increased in the spinal cord, increased 2-fold in the brainstem and approximately 15-fold in the cortex.

At P2, KCC2a contributes approximately half of the total KCC2 in mouse CNS (in brainstem, cortex and spinal cord). KCC2a expression in the P2 brainstem and spinal cord was similar and 3-fold higher than expression in the P2 cortex (Uvarov *et al*, 2009). In adult mouse, KCC2b is the prevalent isoform: the percentage of KCC2a of the total KCC2 is 4% in the cerebellum, 8% in the cortex, 10% in the hippocampus, 11% in the olfactory bulb and 17–18% in the brainstem and spinal cord (Uvarov *et al*, 2009). The amount of the KCC2 protein isoforms in neonatal and adult mouse reflects well their mRNA levels (Uvarov *et al*, 2007).

Using immunohistochemistry, the KCC2b protein was detected mainly in non-cortical regions in rat brain at P4: the olfactory bulb, the rostrodorsal region of the caudate–putamen, thalamus and hypothalamus, the superior and inferior colliculus, the pons, and the medulla. KCC2b protein expression was weak in hippocampus and cortex but some expression was detected in dorsocaudal regions of cortex. In the cerebellum, only ventrocaudal regions demonstrated some KCC2b expression at P4 (Blaesse *et al*, 2006). At P12, KCC2b protein expression was detected at a rather uniform level throughout the brain. In the regions displaying a high KCC2b expression already at P4, there was no additional increase in the signal, whereas in regions with weak KCC2b expression at P4 (hippocampus and most parts of the neocortex and cerebellum), the signal increased until P12 (Blaesse *et al*, 2006).

A similar cellular distribution of KCC2 isoforms was observed by immunohistochemistry in E18 mouse brain (Uvarov *et al*, 2009). KCC2a and KCC2b immunoreactivities were seen in non-cortical regions (including olfactory bulb, basal forebrain, hypothalamus, thalamus, midbrain, and hindbrain), whereas labeling of cerebral cortex and hippocampus was close to the background level. Most neurons in non-cortical brain structures were positive for both KCC2a and KCC2b, although the relative expression of the two isoforms showed some

regional variability. In E18 midbrain double-stained with the KCC2a and KCC2b specific antibodies, most positive neurons were observed to co-express KCC2a and KCC2b (Uvarov *et al*, 2009).

1.3.3 Subcellular localization

In light and electron microscopic studies of mature CNS neurons, KCC2 immunoreactivity is mainly found associated with the plasma membrane in somatic and dendritic compartments. This distribution has been observed in many neuronal cells using antibodies detecting both isoforms, for example in hippocampal pyramidal cells of rat (Gulyas *et al*, 2001, Baldi *et al*, 2010), in neurons of the substantia nigra pars reticulata (Gulacsi *et al*, 2003), in thalamic relay cells (Bartho *et al*, 2004), in mature pyramidal cortical neurons of rat (Szabadics *et al*, 2006), in mouse cerebellar granule cells (Takayama and Inoue, 2006) and in neurons of the suprachiasmatic nucleus of rat (Belenky *et al*, 2008). Similarly, KCC2b isoform immunoreactivity is also clearly concentrated at the plasma membrane of somas and dendrites in spinal motoneurons (Hubner *et al*, 2001, Boulenguez *et al*, 2010, Stil *et al*, 2011) and in brainstem auditory neurons (Blaesse *et al*, 2006).

In thalamic relay cells, the density of KCC2 immunoreactivity was relatively even in the various soma-dendritic compartments and did not correlate with dendritic diameter or synaptic coverage (Bartho *et al*, 2004). Both proximal parts of dendrites as well as distal parts were labeled in the cerebellar granule cells of adult mouse (Takayama and Inoue, 2006). In hippocampal principal neurons of rat, cell type-specific distribution profiles of KCC2 within the dendritic tree were observed: Dendrites of dentate granule cells showed higher KCC2 concentration compared with the soma, but the dendritic distribution was relatively homogeneous. In CA1 pyramidal cells, highest KCC2 density was found in the proximal apical and basal dendrites followed by the somatic membrane, while dendritic region-specific differences were detected between proximal and distal dendrites (Baldi *et al*, 2010).

KCC2 is not detected in axons (Baldi *et al*, 2010, Szabadics *et al*, 2006, Hubner *et al*, 2001). In pyramidal cortical neurons of rat, absence of KCC2 at the axon initial segment was demonstrated as well as a decrease of KCC2 density in the plasma membrane from somatic to axon initial segment (Szabadics *et al*, 2006). In hippocampal principal neurons of rat, the axon initial segment contained very little of KCC2 and the density of KCC2 increases through the somata and towards the dendrites (Baldi *et al*, 2010).

While KCC2 labeling is predominantly observed at the plasma membrane in mature neurons, only little cytoplasmic staining is detected, for example in hippocampal pyramidal cells (Gulyas *et al*, 2001), in neurons of the cochlear nucleus (Vale *et al*, 2005) and neocortex (Szabadics *et al*, 2006). In the cerebellar granule cells of adult mouse, the cytoplasm, Golgi apparatus and endoplasmic reticulum did not show KCC2 labeling (Takayama and Inoue, 2006). In neurons of the suprachiasmatic nucleus, organelles including Golgi apparatus and endoplasmic reticulum occasionally showed KCC2 staining (Belenky *et al*, 2008). In neurons

of the substantia nigra pars reticulata, transport vesicles immunoreactive for KCC2 were occasionally observed in the cytoplasm of dendrites (Gulacsi *et al*, 2003).

In immature neurons, intracellular KCC2 labeling is typically found at a higher level, for example in hippocampal neurons at P2 (Gulyas *et al*, 2001), where KCC2 is seen at the membrane of transport vesicles in dendrites. However, the KCC2b isoform protein was present along the plasma membrane of somata and dendrites in spinal cord motoneurons already at E18.5 similar to adult (Hubner *et al*, 2001). The KCC2b protein was also present at the plasma membrane in early postnatal brainstem auditory neurons (Blaesse *et al*, 2006).

Accumulation of KCC2 was observed in the vicinity of excitatory synapses in hippocampus and in thalamic relay nuclei (Gulyas *et al*, 2001, Bartho *et al*, 2004). In pyramidal neurons of the hippocampus, KCC2 is significantly enriched within dendritic spines (Gulyas *et al*, 2001). KCC2 is also seen near excitatory synapses formed by cerebellar mossy fiber terminals onto granule cells (Takayama and Inoue, 2006).

KCC2 is also observed near symmetrical inhibitory synapses in neurons of the substantia nigra pars reticulata (Gulacsi *et al*, 2003) and the suprachiasmatic nucleus (Belenky *et al*, 2008) where KCC2 was observed to colocalize with GABA type A receptors. The distribution profile of KCC2 along apical dendrites in rat hippocampal CA1 neurons correlated well with the distribution of GABAergic synapses (Baldi *et al*, 2010).

The KCC2b isoform was also enriched near inhibitory synapses in adult spinal motoneurons and colocalized with gephyrin, a postsynaptic protein involved in the clustering of glycine and GABAA receptors (Hubner *et al*, 2001). In neurons of the auditory branstem KCC2b was observed both near excitatory and inhibitory synapses (Blaesse *et al*, 2006).

In immunostainings, KCC2 in several studies shows a punctate distribution at the plasma membrane (Blaesse *et al*, 2006, Gulyas *et al*, 2001, Belenky *et al*, 2008), and this was suggested to correspond to functional tyrosine-phosphorylated KCC2 localized in lipid raft microdomains in cultured rat hippocampal neurons (Watanabe *et al*, 2009). Similarly, KCC2 immunoreactivity in mature rat brain was localized both to membrane rafts and non-raft domains (Hartmann *et al*, 2009). In neonatal rat brainstem, KCC2 largely partitioned into membrane rafts and was found to be in an inactive form, while an increase in KCC2 clustering and transport activity was observed in the absence of lipid rafts (Hartmann *et al*, 2009).

1.3.4 Functional roles of KCC2 isoforms

KCC2 function determines the efficacy and polarity of the chloride-permeable GABA type A and glycine receptor mediated synaptic transmission. Early in development, the expression and activity of KCC2 in neurons is low relative to NKCC1, resulting in a high intracellular Cl^- concentration and depolarizing responses to GABA and glycine. As neurons mature,

KCC2 expression and activity is upregulated, resulting in a reversal of the Cl^- electrochemical potential in neurons and GABA and glycine responses become hyperpolarizing (Rivera *et al*, 1999).

In several areas of neonatal mouse brain, KCC2 protein expression levels appear high but responses to GABA are still depolarizing (Khirug *et al*, 2010). KCC2 seems to be transport-inactive in early development for example in neonatal brainstem neurons (Blaesse *et al*, 2006, Balakrishnan *et al*, 2003), and in immature hippocampal neurons (Khirug et al 2005). The functional activation of KCC2 in development is suggested to involve transport to the plasma membrane, oligomerization, phosphorylation/dephosphorylation or other protein modifications (Blaesse *et al*, 2006, Zhang *et al*, 2006, Khirug *et al*, 2005, Friedel *et al*, 2015).

KCC2 null mutant mice that completely lack both KCC2 isoforms show a disrupted breathing rhythm and die immediately after birth (Hubner *et al*, 2001, Tornberg *et al*, 2005). The respiratory failure is due to a disrupted inspiratory-related rhythmic motor output of the brainstem pre-Bötzinger complex (Hubner *et al*, 2001). Motoneurons of the spinal cord also showed an excitatory response to GABA or glycine, whereas in wild-type mice of the same age an inhibitory response was observed. Expression of KCC2 mRNA and protein is high in mouse brainstem and spinal cord neurons already at birth (Hubner *et al*, 2001, Balakrishnan *et al*, 2003) and the function of KCC2 in these brain regions thus seems to be important already at birth.

KCC2 hypomorph mice, which express only about 20% of KCC2 are viable and fertile and have normal locomotor activity and motor coordination but exhibit a growth deficit and increased anxiety-like behavior (Tornberg *et al*, 2005).

Selective KCC2b isoform knock-out mice, in which the KCC2b isoform has been disrupted leaving the expression of the KCC2a isoform untouched, can survive up to three weeks after birth (Woo *et al*, 2002). These mice appear normal at birth but demonstrated abnormal posture and gait and frequent spontaneous seizures within a couple of days after birth that ultimately led to their deaths (P12–P17). The KCC2a expression in the brain and spinal cord (~50% of total KCC2 in newborn mice) is thus presumably enough to allow the mice to bypass the lethality observed with full KCC2 KO mice at birth.

Recordings from KCC2b KO mice neurons have confirmed the important role of the KCC2b isoform for chloride homeostasis: In dissociated cortical neurons derived from KCC2b-deficient mice, no significant decrease in the intracellular chloride concentration was observed after three weeks in culture in contrast to wild-type neurons (Zhu *et al*, 2005). Neonatal spinal motoneurons of KCC2b-deficient mice (Stil *et al*, 2011) demonstrated a more depolarized glycine reversal potential similar to that found in motoneurons of KCC2 null mice (Hubner *et al*, 2001).

Cultured cortical neurons from KCC2 null mice demonstrate long filopodia-like spines and a reduced number of functional excitatory synapses and reduced mEPSC frequency (Li *et al*, 2007). Spine maturation could be rescued by expression of a non-functional, N-terminal deficient KCC2 and mimicked by expression of the KCC2 C-terminal domain (Li *et al*, 2007).

Since the KCC2 C-terminal region interacts with protein 4.1N which is an actin-interacting protein important in cytoskeletal organization at synapses, this interaction may contribute to maturation of spines (Li *et al*, 2007). Supporting this, premature expression of KCC2 in mouse cortex induces an increase in spinogenesis and excitatory synapse density *in vivo* (Fiumelli *et al*, 2013). It has also been shown that the KCC2 interaction with 4.1N is important for the plasticity of AMPA receptors in mature neurons and thus for the basal excitatory activity at mature cortical excitatory synapses (Gauvain *et al*, 2011).

Hippocampal networks in KCC2 null mice demonstrate changes in activity, hyperexcitability and generate spontaneous seizure activity at E18.5 (Khalilov *et al*, 2011). More GABAergic and glutamatergic synapses and currents were also observed in the KCC2 null embryos compared to wild-type embryos. However, KCC2 was not functional as a transporter at this time-point, as no significant difference in the intracellular Cl^- concentration was observed between hippocampal neurons from KCC null and wild type mice. These results thus suggest that a transport-unrelated functions of KCC2 are important already at embryonic stages (Khalilov *et al*, 2011).

In cortical structures KCC2 labeling is first detected at the end of embryonic development and peaks during the second postnatal week (Blaesse *et al*, 2006, Stein *et al*, 2004). Pyramidal cortical neurons show very little KCC2 expression at birth and during the first postnatal week. However, in the developing mouse cortex, in a subset of tangentially migrating interneurons, KCC2 has been detected already at the time of birth, prior to synaptogenesis (Bortone and Polleux, 2009). When KCC2 is upregulated in these neurons, the ambient GABA mediated depolarization becomes hyperpolarizing, acting as a stop signal for the interneurons, leading to termination of migration (Bortone and Polleux, 2009).

1.4 THE WNK–SPAK/OSR1 PATHWAY

Ion transport mediated by CCCs across the cell surface is accompanied by water flux and thus CCCs also participate in maintaining water homeostasis (osmoregulation) (MacAulay *et al*, 2004). WNK kinases (With No lysine =K) and their downstream kinases SPAK/OSR1 of the Ste20- family are serine-threonine kinases and key regulators of CCCs in response to osmotic challenges (Kahle *et al*, 2010, Alessi *et al*, 2014). It has also been shown that the WNK-SPAK/OSR1 pathway is directly regulated by intracellular Cl^- as the activation of WNKs is prevented when Cl^- binds to the active site and stabilizes the inactive conformation (Piala *et al*, 2014).

Exposure of HEK293 cells to conditions that activate the WNK signalling pathway resulted in increased phosphorylation of KCCs, WNK1, SPAK/OSR1 and NKCC1, while conditions that inhibit the WNK signalling pathway induced a rapid dephosporylation of the same molecules (de Los Heros *et al*, 2014).

Stimuli such as extracellular hyperosmotic challenge or intracellular chloride decrease leads to activation of the WNK–SPAK/OSR1 pathway and results in serine/threonine phosphorylation of CCCs. Phosphorylation of CCCs via WNK–SPAK/OSR1 pathway activates the transport function of N(K)CCs and inhibits that of KCCs. Activity of N(K)CCs results in a net influx of chloride and water that compensates for cell shrinking and results in a regulatory volume increase. On the other hand, hypoosmotic challenge, hypotonic high K+ conditions or a rise in internal chloride concentration results in inhibition of the WNK–SPAK/OSR1 pathway and dephosphorylation of CCCs. Dephosphorylation inhibits N(K)CCs and activates KCCs and results in a net efflux of chloride and water and a regulatory volume decrease acting to compensate cell swelling (Alessi *et al*, 2014).

In response to activated WNK-SPAK/OSR1 pathway KCC2 is phosphorylated at T906 (termed site1) and T1007 (termed site2) in the C-terminal domain (Rinehart *et al*, 2009). When overexpressed in HEK293 cells, KCC2 is robustly phosphorylated at Thr906 and Thr1007 and dephosphorylation of these sites significantly stimulates KCC2 activity (Rinehart *et al*, 2009, de Los Heros *et al*, 2014). Dual dephosphorylation of KCC2 at Thr906 and Thr1007 also strongly stimulates KCC2 activity in neurons (Kahle *et al*, 2013, Friedel *et al*, 2015). Site1 and site2 are conserved in all KCCs and the phosphorylation/dephosphorylation has similar effects on all KCCs (Rinehart et al., 2009). SPAK and OSR1 are known to directly phosphorylate site2 of KCCs, but they may not phosphorylate directly site1 (de Los Heros *et al*, 2014).

In addition to site2, multiple other serine (S78, S83) and threonine (T6, T77, T92) residues in the KCC2a isoform have been shown to be directly phosphorylated by SPAK kinase *in vitro* (de Los Heros *et al*, 2014). All of these sites except T6, which is located in the KCC2a isoform specific N-terminal part, are also present in the KCC2b isoform (see Fig 4). Moreover, the T6 site was found to be conserved in all KCCs except the KCC3A isoform (de Los Heros *et al*, 2014).

KCC-mediated ion transport is induced by cell swelling and downregulated at low intracellular Cl^- concentration (Adragna *et al*, 2004, Lytle and McManus, 2002). When expressed in Xenopus oocytes, KCC1, KCC3, and KCC4 express minimal activity in isotonic conditions but are strongly activated by cell swelling induced by hypotonic conditions (Mercado *et al*, 2000). KCC2 was found to be functional at isotonic conditions when expressed in Xenopus oocytes and exhibited minimal activation by cell swelling (Strange *et al*, 2000), likely due to the ISO domain (Mercado *et al*, 2006). However, the transport activity of human KCC2 overexpressed in Xenopus laevis oocytes was stimulated by cell swelling (Song *et al*, 2002). Activation of K+-Cl– cotransport by cell swelling has been ascribed to the inhibition of the WNK-SPAK/OSR1 kinases, combined with the activation of protein phosphatase 1 and 2A -dependent dephosphorylation (Kahle *et al*, 2015).

The members of WNK family have been shown to inhibit the activity of the KCC2 cotransporter exogenously overexpressed in Xenopus oocytes (de Los *et al*, 2006, Gagnon *et al*, 2006, Rinehart *et al*, 2011). The transport function of KCC2 was diminished by the expression of SPAK and WNK4 under both isosmotic and hyposmotic conditions (Gagnon *et al*, 2006). WNK1 inhibited the activity of all mammalian KCCs (KCC1, KCC2a, KCC2b, KCC3a, KCC3b, and KCC4) when coexpressed in Xenopus oocytes (Mercado *et al*, 2016).

KCC3 is expressed in multiple tissues, and knockout studies have revealed that it is important in volume regulation and particularly in neuronal volume regulation following hyposmotic swelling (Rust *et al*, 2006, Boettger *et al*, 2003, Byun and Delpire, 2007). In contrast, the primary physiological function of the constitutively active KCC2 is to maintain the low intracellular Cl^- concentration of neurons in isotonic conditions. However, results suggest that KCC2 may also participate in osmotic and volume regulation in dendritic spines following isosmotic activity-induced neuronal swelling (consisting of an increase in the net influx of Na^+, Cl^- and osmotically obliged water) (MacAulay *et al*, 2004, Jourdain *et al*, 2011, Gauvain *et al*, 2011).

The phosphorylation state of KCC2 is correlated with its functional activation during development (Khirug *et al*, 2005) and the WNK-kinase pathway might be involved in the developmental activation of KCC2. The KCC2 Thr906 and Thr1006 residues are phosphorylated at early stages of development and their dephosphorylation parallels the maturation of GABAergic transmission (Kahle *et al*, 2013, Rinehart *et al*, 2009). WNK1 dependent phosphorylation of KCC2 in mature neurons causes a loss of transport function (Rinehart *et al*, 2009, Kahle *et al*, 2005). Thr-906 and Thr-1007 phosphorylation of KCC2 did not affect cell surface KCC2 expression of KCC2 (Rinehart *et al*, 2009). Overexpression of active WNK1 also resulted in KCC2 inhibition in cultured neurons (Inoue *et al*, 2012). Knockdown of WNK1 in immature neurons resulted in dephosphorylation of Thr906 and Thr1007 of KCC2 and significantly enhanced KCC2-dependent Cl^- extrusion and caused a hyperpolarizing shift of the EGABA (Friedel *et al*, 2015).

SPAK/OSR1 interact with RFx(V/I) motifs present in N-terminal domains of CCCs (Delpire and Gagnon, 2008, Richardson *et al*, 2008). The RFx(V/I) motif is present in N-terminal domains of several CCC family isoforms; at least one splice variant of each

mammalian CCC family member, except KCC1, has the binding motif. The N-terminal domain of the KCC2a isoform contains this sequence, but it is not present in the KCC2b isoform (Uvarov *et al*, 2007). The motif is also found in N-termini of KCC2 orthologs in Drosophila (Hekmat-Scafe *et al*, 2006) and C. elegans (Tanis *et al*, 2009).

RFx(V/I) motifs are also present on WNK isoforms and SPAK kinases are known to interact with WNK kinases through this motif (Piechotta *et al*, 2003). Elimination of the SPAK/OSR1binding site of WNK1 prevented the effect of WNK1 on KCCs suggesting that activation of WNK1 depends on the interaction with SPAK /OSR1 (Mercado *et al*, 2016).

It has also been suggested that WNK kinases interact with each other by oligomerization, and the WNK-WNK interaction was necessary for the WNK1 inhibitory effect on KCCs (Mercado *et al*, 2016). KCC2 has also been shown to form a physical complex with WNK1 kinase in the developing brain, and this interaction might function as a link between SPAK and KCC2 (Friedel *et al*, 2015).

2. AIMS OF THIS STUDY

The general aim of this work was to elucidate the isoform-specific differences in the expression, function and regulation of the KCC2 isoforms. More specific goals were to study the following:

1. The involvement of transcription factors USF1 and USF2 in the KCC2b transcriptional regulation via the E-box motif.

2. The expression patterns and subcellular localization of the KCC2a and KCC2b isoforms in mouse CNS using immunohistochemistry.

3. The functionality of the KCC2a isoform as a chloride cotransporter in neurons.

4. The binding and functional regulation of the KCC2 isoforms by SPAK.

3. MATERIALS AND METHODS

The experimental methods used in this work are listed in Table I with a reference to the appropriate original publication where detailed descriptions can be found. Transgenic mice are listed in Table II and expression constructs are listed in Table III. Primary antibodies are discussed in section 3.1, and listed in Table IV.

Table I. Methods used and described in the original articles

Method	**Used in**
Western blotting (WB)	I,II,III
Electrophoretic mobility shift assay (EMSA)	I
Chromatin immunoprecipitation assay (ChIP)	I
Cell culture	I,II, III
Transfection	II, III
Animals and tissue processing	II, III
Immunohistochemistry (IHC)	II,III
In situ hybridization (ISH)	II
Microscopy, imaging	II, III
Immunocytochemistry (ICC)	III
Electrophysiological recordings	III
Calcium imaging	III
Biotinylation assay	III
Coimmunoprecipitation assay (coIP)	III
Functional ^{86}Rb flux assay	III

Table II. Transgenic Mice

Strain	**Description**	**Source/Reference**	**Used in**
KCC2null	Knock-out mice that lack both KCC2 isoforms	(Tornberg *et al*, 2005)	II,III
KCC2a-KO	Selective KCC2a isoform knock-out mice	Study II	II
KCC2b-KO	Selective KCC2b isoform knock-out mice	(Woo *et al*, 2002)	III

Table III. Plasmid constructs

Construct	Description	Source/Reference	Used in
KCC2b(0.6)	0.6 kb KCC2 promoter fragment in pGL3-Basic (Promega) firefly luciferase reporter vector.	Study I	I
E-box mut	KCC2b(0.6) construct with a mutation in the E-box motif	Study I	I
A-USF	USF dominant-negative mutant in modified CMV566 expression vector	Dr. Charles Vinson (Qyang *et al*, 1999)	I
KCC2a	Full-length rat KCC2a cDNA in pcDNA3.1 (Invitrogen) expression vector	(Uvarov *et al*, 2007)	III
KCC2b	Full-length rat KCC2b cDNA in pcDNA3.1 (Invitrogen) expression vector	(Uvarov *et al*, 2007)	III
HA-SPAK	Hemagglutinin (HA) –tagged SPAK	Dr. Forbush (Dowd and Forbush, 2003)	III
DNSPAK	Dominant negative, kinase-inactive K101R form of SPAK	Dr. Forbush (Dowd and Forbush, 2003)	III

3.1 PRIMARY ANTIBODIES

The KCC2a antiserum was raised in rabbit against a 21 -amino acid peptide corresponding to the N-terminus of the mouse KCC2a sequence (amino acids 20-40) (Uvarov *et al*, 2009) (Fig. 5). The specificity of the anti-KCC2a antiserum was tested in Western blot and immunohistochemistry using neonatal wild-type and KCC2null mutant mice (Uvarov *et al*, 2009). The antibody produced a high background in immunostainings of mouse brain cryosections when using 4% paraformaldehyde (PFA) fixation, but the background was significantly decreased when using cold methanol-acetone (1:1) fixation (Uvarov *et al*, 2009). The KCC2a antiserum was also tested in immunocytochemistry with hippocampal neurons derived from E17 wild-type and KCC2 null KO mice embryos (Uvarov *et al*, 2009), and the antiserum produced a specific staining when using methanol-fixation of the cells.

In study II we set out to optimize the immunostaining method with the KCC2a antiserum and PFA-fixed tissue. Prior to use in immunostainings, the KCC2a antiserum was preabsorbed against PFA-fixed tissue from KCC2a-KO mice. This helped to reduce the background signal in immunostainings with PFA-fixed adult mouse brain sections (Study II, Fig 1). Moreover, shorter post-fixation time with 4% PFA (30 minutes or 2 hours at most) also reduced the nonspecific signal. Preabsorbed antiserum and short post-fixation time was thus used with PFA-fixed adult mouse sections in study II and study III. Preabsorption was less efficient at removing the background staining from PFA-fixed young postnatal mouse

brain sections in KCC2a immunohistochemistry, thus methanol–acetone post-fixation was used for the earlier time points in study II.

The KCC2b antiserum was raised in rabbit against a 15-amino-acid peptide corresponding to the N-terminus of the KCC2b isoform (amino acids 8-22) (Hubner *et al*, 2001) (Fig. 5). The last five amino acids in this peptide are common to both KCC2 isoforms, but the antibody is highly specific for the KCC2b isoform (Uvarov *et al*, 2009). In study II we generated KCC2b antibodies against the same peptide in chicken, and the specificity of the antibodies was tested in Western blot using brain lysates from wild-type and KCC2 null mice (Study II, Fig. 2).

The KCC2-pan antiserum was generated in rabbit against a C-terminal peptide of rat KCC2 (amino acids 932–1043), corresponding to a region that is highly variable among KCCs (Williams *et al*, 1999, Ludwig *et al*, 2003) (Fig. 5). The specificity of the antibody has been tested using wild-type and KCC2 null mice brain homogenates in Western blot analysis (Ludwig *et al*, 2003), and in immunohistochemistry (Uvarov *et al*, 2009). A monoclonal mouse KCC2pan antibody against the same C-terminal peptide of rat KCC2 has also been generated (Chemicon /Millipore), the antibody was tested in immunohistochemistry in study II, and it produced an identical staining pattern as the rabbit KCC2pan antibody.

Table IV. Primary antibodies used in this work

Against	**Host**	**Source/Reference**	**Methods**	**Used in**
USF1	Rb	Santa Cruz	EMSA, ChIP	I
USF2	Rb	Santa Cruz	WB, EMSA, ChIP	I
Egr4	Rb	(Zipfel *et al*, 1997)	EMSA	I
KCC2a	Rb	(Uvarov *et al*, 2009)	WB, IHC	II, III
KCC2b	Ch	study II	WB, IHC	II, III
KCC2b	Rb	(Uvarov *et al*, 2009, Hubner *et al*, 2001)	IHC	II
KCC2pan	Rb	(Williams *et al*, 1999, Ludwig *et al*, 2003)	WB, IHC,	II, III
KCC2pan	Ms	Chemicon /Millipore	IHC	II
MAP2	Ms	Chemicon /Millipore	IHC	II
SPAK	Rb	(Ushiro *et al*, 1998)	WB, coIP	III
β-tubulin	Ms	BabCO	WB	III
HA	Ms	GE Healthcare	WB, coIP	III

Ch (chicken), Ms (mouse), Rb (rabbit), MAP2 (microtubule-associated protein 2), HA (hemagglutinin)

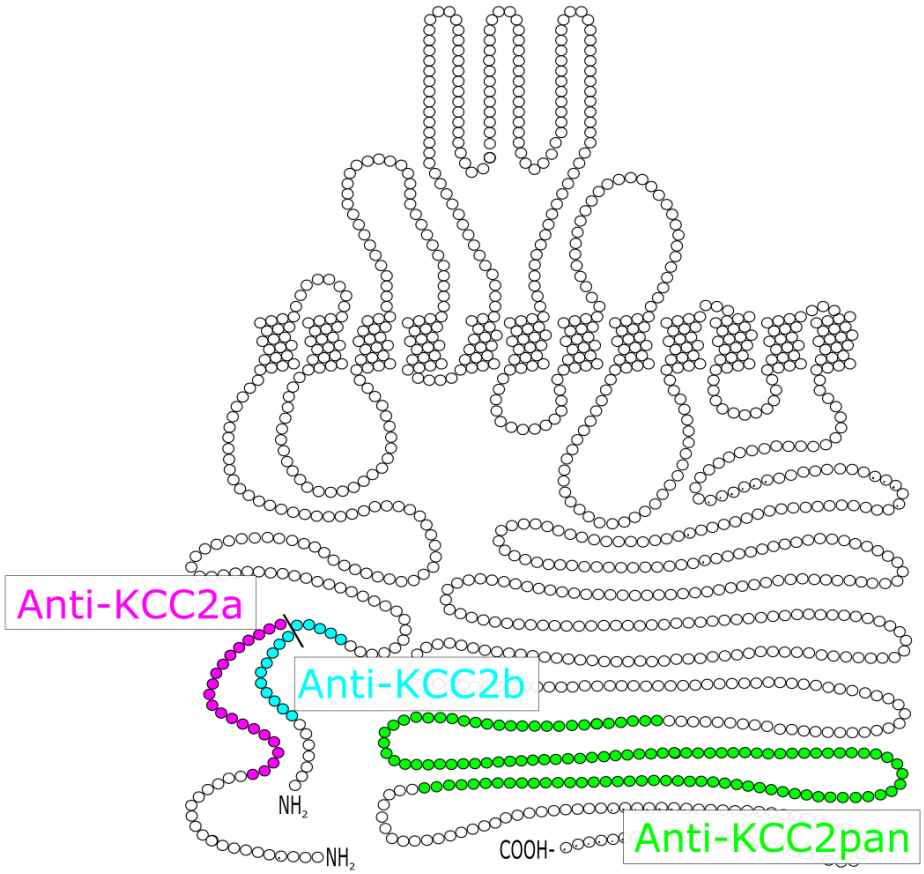


Fig. 5. KCC2 antibodies
Shown are the peptides against which selected KCC2 antibodies have been generated. The KCC2a antiserum was raised against a 21 -amino acid long peptide in the KCC2a isoform specific N-terminal part. KCC2b antibodies have been generated in rabbit and chicken against a 15-amino acid long sequence in the N-terminal part of the KCC2b isoform. Five amino acids of this peptide belong to the part of N-terminus that is common to both isoforms. KCC2pan antiserum was generated in rabbit against a 112-amino acid long segment in the common C-terminal part of KCC2, and the KCC2pan antibodies thus detect both KCC2 isoforms.

4. RESULTS AND DISCUSSION

4.1 REGULATION OF KCC2B EXPRESSION BY USF PROTEINS

Sequence analysis of the mouse KCC2b promoter region showed that the -319 to -314 - region contained an E-box consensus element (CACGTG) (Uvarov *et al*, 2006). The E-box element was conserved in the mouse, rat, human and chimpanzee KCC2b promoters. The E-box element is a hexanucleotide sequence (CANNTG) recognized by the basic helix-loop-helix (bHLH) family of transcription factors (Jones, 2004). Many bHLH proteins bind strongly to the CG dinucleotide containing palindromic E-box CACGTG.

Members of the bHLH family are found in almost all eukaryotes (Jones, 2004). The bHLH domain comprises a DNA-binding basic region (b) and a helix-loop-helix (HLH) motif, which is involved in homo- and hetero- dimerization with other HLH proteins. The two basic domains brought together through dimerization bind the transcription factor to DNA and each monomer of the bHLH dimer binds a CAN half-site. A number of bHLH protein families have a leucine-zipper domain that also mediates dimerization in addition to the HLH motif (Jones, 2004).

Upstream stimulating factors 1 (USF1) and 2 (USF2) are bHLH/leucine zipper transcription factors that bind to E-box elements (Sirito *et al*, 1994). USFs were initially identified as a cellular transcription factor for the *adenovirus-2 major late* gene (Carthew *et al*, 1985, Moncollin *et al*, 1986, Sawadogo and Roeder, 1985). USFs normally bind as USF1/USF2 heterodimers to the E-box element and are involved in transcriptional activation. Both USF1 and USF2 transcription factors are ubiquitously expressed in animal tissues (Sirito *et al*, 1994, Sirito *et al*, 1998) but the expression level in brain is particularly high (Fujimori and Urade, 2007, Park and Russo, 2008). USFs normally bind to the palindromic consensus E-box sequence, CACGTG (Sawadogo and Roeder, 1985). Electrophoretic mobility shift assay (EMSA) and supershift experiments have indicated that USF proteins are the major binding factors for the palindromic CACGTG E-box motif in mouse brain (Yamanaka *et al*, 2016).

A large number of USF target genes has been identified, and include many genes important for fatty acid synthesis and insulin signaling (Corre and Galibert, 2005, Rada-Iglesias *et al*, 2008). The complete knockout of USF1 and USF2 induces embryonic lethality, demonstrating the requirement of USFs during embryogenesis (Sirito *et al*, 1998) . The knockout of USF2 suppresses glucose-dependent gene expression in mouse liver (Vallet *et al*, 1997), and USF1 has been associated with familial combined hyperlipidemia (Pajukanta *et al*, 2004).

USFs also play essential roles in normal brain function: Both USF1 and USF2 knockout mice display a propensity for spontaneous epileptic seizures (Sirito *et al*, 1998). USFs have also been shown to regulate the expression of several neuronal genes, for example, fragile X mental retardation 1 (FMR1) (Kumari and Usdin, 2001), BDNF (Chen *et al*, 2003, Tabuchi *et*

al, 2002) amyloid precursor protein (Hoffman and Chernak, 1995), GABA B1 receptor (Steiger *et al*, 2004), and prodynorphin (Bazov *et al*, 2017).

In study I, we used EMSA and supershift assay with specific antibodies to USF1 and USF2, in order to determine if USF proteins associated with the E-box element of the KCC2b promoter *in vitro* (Study I, Fig 1). Our results showed that USF1 and USF2 proteins specifically bound to the E-box of KCC2b *in vitro* when using nuclear protein lysates of both mouse neuroblastoma N2a cells and NIH3T3 fibroblasts. The supershift assay demonstrated, that USF1/USF2 heterodimers appeared to form the major E-box binding complex in our experiment.

Subsequently, the functional role of the E-box motif and USF transcription factors were examined in neuroblastoma N2a and NIH3T3 cells using a luciferase reporter assay (Study I, Fig 2). Cells were transiently transfected with a luciferase reporter construct containing a 0.6 kb KCC2b promoter fragment (KCC2b(0.6)). Mutation of the E-box element in the KCC2b promoter (E-box mut construct) reduced the activity of the KCC2b promoter, suggesting that the E-box element is involved in basal KCC2b gene expression. Co-transfection with a dominant-negative form of USF (A-USF) was used in order to confirm the involvement of endogenous USF proteins in the regulation of the KCC2b gene. The A-USF functions by specifically dimerizing with USF1 and USF2 and preventing their binding to DNA (Qyang *et al*, 1999). Overexpression of A-USF resulted in supression of the KCC2b(0.6) reporter activity, indicating a contribution of endogenous USF proteins to expression driven by the KCC2b promoter in N2a and NIH3T3 cells.

The role of the E-box motif of the KCC2b promoter, as well as binding of the USFs, was also studied in neurons (Study I, Fig 3). Evidence that endogenous USF1 and USF2 bind the KCC2b promoter was provided by chromatin immunoprecipitation assay (ChIP) in rat primary cortical neurons (8 DIV). The role of the E-box motif was also examined in a luciferase reporter assays in primary cortical cultures transfected with the KCC2b(0.6) construct. Mutation of the E-box element in the KCC2b promoter region (E-box mut construct) reduced the activity of the KCC2b promoter in 6 DIV cortical neurons by ~23%, but in 10 DIV cortical neurons, there was no significant change in promoter activity. The results suggested that the E-box element is involved in basal KCC2b gene expression in immature cortical neurons.

The activity of USF proteins, such as DNA binding activity and transactivation effects, are modulated by many signaling cascades in cells (Galibert *et al*, 2001, Cheung *et al*, 1999, Nowak *et al*, 2005, Xiao *et al*, 2002). Post-translational modifications like phosphorylation have been shown to regulate the function of USFs, and USFs are phosphorylated by various kinases (Horbach *et al*, 2015). Among the protein kinases that phosphorylate USF1 are the cyclin dependent kinase cdk2, protein kinase A, phosphatidylinositol 3-kinase, DNA-dependent protein kinase (DNA-PK), stress activated kinase p38, and ERK1/2 kinase. Only a few kinases such as protein kinase A (PKA) and GSK3β are known to phosphorylate USF2 (Horbach *et al*, 2015).

Interactions with other transcription factors or co-factors have also been shown to regulate the USF-dependent transactivation. Among the transcription factors and co-factors that have been described to interact with USFs are the b-Zip family protein Fra1 (Pognonec *et al*, 1997), the bHLH transcription factor Cha (Rodriguez *et al*, 2003), the hypoxia-inducible transcription factor HIF2α (Befani *et al*, 2013), the CCAAT/enhancer binding protein C/EBPβ (Dahle *et al*, 2002), and the homeodomain of the pancreatic transcription factor PDX-1 (Amemiya-Kudo *et al*, 2011).

In neurons, USF proteins have been particularly linked to activity-dependent regulation of transcription by Ca^{2+}-activated signaling pathways: A Ca^{2+}-responsive composite E-box/cAMP-responsive element (CRE) site was bound by USF1 and USF2 in the *BDNF* promoter I (Tabuchi *et al*, 2002), and in the *BDNF* promoter III a Ca^{2+}-responsive E-box element (CaRE) was bound by USF1 and USF2 (Chen *et al*, 2003). USFs also bound to a depolarization-sensitive composite CREB/Activating transcription factor-4 (ATF4)/USF regulatory element in the *GABA B1* receptor promoter (Steiger *et al*, 2004).

An E box element bound by USFs in the rat *preprotachykinin A* promoter regulated the activity of this promoter in response to nerve growth factor (NGF) in dorsal root ganglion neurons (Gerrard *et al*, 2005).

The expression level of USFs in brain is particularly high (Fujimori and Urade, 2007, Park and Russo, 2008), but the cellular expression pattern of USF1 and USF2 is not completely overlapping when studied in immunohistochemistry (Yamanaka *et al*, 2016): USF1 expression was strong in cortical neurons but relatively weak in hippocampus and striatal neurons, whereas USF2 was expressed in neurons of these brain regions to almost the same level. In another study, substantial cell-to-cell differences in USF2 expression were detected in the dorsolateral prefrontal cortex in immunohistochemistry (Bazov *et al*, 2017), and it was also reported that neurons and non-neuronal cells expressed USF2 at similar levels (Bazov *et al*, 2017).

The palindromic E-box motif (CACGTG) contains a potential CpG methylation site, and USFs have been identified as methylation-sensitive transcription factors (Griswold and Kim, 2001). Expression of the *prodynorphin* gene was associated with differential methylation of its promoter between neurons and glia (Bazov *et al*, 2017): The promoter was hypomethylated in neurons and hypermethylated in non-neuronal cells. USF2 bound to unmethylated E-box in the promoter of the *prodynorphin* gene, and activated gene transcription. Methylation of the E-box prevented the activation of *prodynorphin* transcription by USF2. In EMSA assay USF2 bound to a nonmethylated E-box oligonucleotide with significantly higher affinity compared to a methylated E-box oligonucleotide (Bazov *et al*, 2017).

Our results suggested that transcription factors binding to the E-box element in the KCC2b promoter participate in the basal KCC2b gene expression in immature neurons and could thus be involved in the developmental upregulation of KCC2b *in vivo*. Since the KCC2b promoter in rat primary cortical neurons at DIV8 was clearly occupied by USF1 and USF2 in ChIP assay, this would suggest that USF1/USF2 heterodimers are involved in the

regulation of KCC2b via the E-box motif. This conclusion is also supported by our luciferase reporter assays in cell lines. However, as the binding and activity of USFs depends on various signalling pathways and interactions with other transcription factors or co-factors it is also possible that USFs participate in regulation of KCC2b transcription in response to yet unidentified conditions.

USFs have been reported to function together with Ca^{2+} responsive transcription factors, such as CREB, in neurons (Chen *et al*, 2003, Steiger *et al*, 2004, Tabuchi *et al*, 2002), and this could be important also in the regulation of KCC2b. Activity- dependent influx of Ca^{2+} via voltage-gated calcium channels has been suggested as one mechanism of KCC2 upregulation in development (Ganguly *et al*, 2001). In mature hippocampal neurons, BDNF-TrkB signaling resulted in a down regulation of KCC2 gene expression via activation of the PLCγ pathway and the transcription factor CREB (Rivera *et al*, 2004). However, no binding sites for CREB or calcium-responsive transcription factor (CaRF) have been identified in the KCC2b promoter.

Downregulation of KCC2 mRNA has been observed in epilepsy (Palma *et al*, 2006, Huberfeld *et al*, 2007) and sustained interictal-like activity resulted in a down regulation of KCC2 gene expression via the the BDNF-TrkB signaling cascade and CREB (Rivera *et al*, 2004, Rivera *et al*, 2002). Since both USF1 and USF2 knockout mice were prone to epileptic seizures (Sirito *et al*, 1998), decreased activity of USFs could be one putative mechanisms involved in the downregulation of KCC2b mRNA in epilepsy.

In immature neurons, activation of TrkB by BDNF was shown to trigger only the Shc pathway resulting in the activation of a number of adaptor proteins and in the GTP-loading of Ras and subsequently to the activation of the mitogen-activated protein (MAP) kinase cascade. Sequential phosphorylation of Raf, Mek, and ERK, and translocation of ERK the nucleus. ERK1/2 activation resulted in the upregulation of KCC2 transcription via transcription factor Egr4 (Ludwig *et al*, 2011). ERK1/2 have also been shown to phosphorylate and activate USFs (Imagawa *et al*, 2006, Park and Russo, 2008). For example, in trigeminal ganglion neurons, USFs were found to be downstream targets of the MAP kinase and ERK pathway (Park and Russo, 2008).

Since NRSE sites seem not to be critical for the neuron-specific expression of KCC2, it is possible that transcription factors binding to the KCC2b promoter region are sufficient to drive the neuron-specific expression of KCC2b mRNA (Uvarov *et al*, 2005). In line with this, the KCC2b promoter region contains several predicted binding sites for neuron-enriched transcription factors such as AP2, AP1, Mef2 and Egr4 (Uvarov *et al*, 2006). For example, Egr4 is highly enriched in neurons and has a similar temporal expression pattern (Uvarov *et al*, 2006). However, it has also been shown that USFs may direct neuron specific transcription even though they are ubiquitously expressed transcription factors (Bazov *et al*, 2017). The cellular environment is important in coordinating various cell-type specific functions of USFs and could be mediated by epigenetic factors, such as DNA methylation (Bazov *et al*, 2017).

As there are many E-box binding bHLH transcription factors, the effects of other bHLHs cannot be completely excluded. Several bHLH proteins are widely expressed in the CNS, for example c-Myc, Max, Neuro D. As binding and activity of USFs depends on interactions with other transcription factors, co-factors and the chromatin structure, other bHLH may associate with the E-box element of the *KCCC2b* promoter in another cellular context. A hypothetical model of the transcriptional regulation of KCC2b gene is presented in Fig. 7.

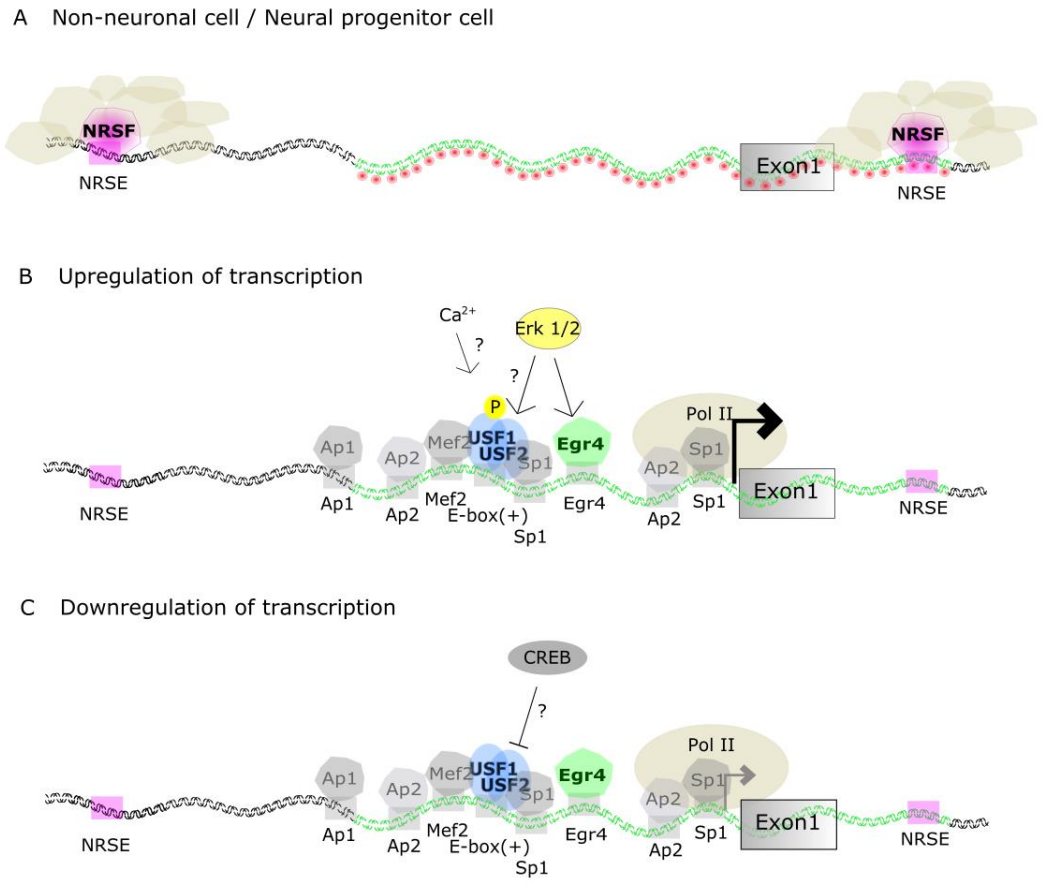


Fig. 6 Transcriptional regulation of KCC2b gene, a putative scenario.
A. KCC2b gene promoter in non-neuronal or neural progenitor cells. The green DNA strand represents a CpG island with methylation (red dots). NRSF is bound to NRSE sites in the 5' regulatory region of KCC2b and in intron 1b. Co-repressor complexes are associated with the NRSFs and participate in the covalent modification of chromatin. Activating transcription factors are unable to bind and initiate transcription.
B. In immature neurons, in the absence of DNA methylation, activating transcription factors bind to the DNA elements in the KCC2b promoter region. USF1/USF2 heterodimers and Egr4 are shown as well as other putative transcription factors (Sp1, AP2, AP1, Mef2). RNA polymerase II binds and initiates the transcription. ERK1/2 kinases as well as Ca^{2+}-activated signaling pathways might be important in the upregulation of KCC2b transcription in development and could function in the activation of USF proteins, for example by stimulating their phosphorylation.
C. In mature neurons, downregulation of KCC2b transcription can take place, for example the activity-dependent downregulation mediated by the transcription factor CREB. The binding site of CREB is not known, but USFs have been reported to function together with CREB and perhaps could be involved in the transcriptional repression of KCC2b mediated by CREB.

4.2 CELLULAR EXPRESSION PATTERN

In study II we compared the expression pattern of KCC2a and KCC2b in different regions of postnatal mouse CNS by immunohistochemistry. Several different developmental time points were analyzed: E18, P3, P7, P12, and adult. In addition, some immunostainings from hippocampal formation in adult mouse was performed in study III. Rabbit anti KCC2pan and KCC2 isoform specific antibodies (rabbit anti-KCC2a, chicken anti-KCC2b and rabbit anti-KCC2b) were used in these studies. Double-stainings with the isoform specific antibodies raised in different species (rabbit vs. chicken) were performed in order to detect KCC2a and KCC2b in the same section.

In study II, the nonspecific staining produced by the KCC2a antiserum in PFA-fixed brain sections (Uvarov *et al*, 2009) was reduced significantly when KCC2a antiserum was preabsorbed against brain sections from PFA-fixed KCC2a-KO mice. We tested the specificity of the KCC2a antiserum by staining sections of adult wild-type and KCC2a-KO mice perfusion-fixed with 4% PFA (Study II, Fig 1). In study II and III, immunohistochemical staining using the KCC2a antiserum with PFA-fixed adult sections were performed by using preabsorbed antibodies. However, methanol-acetone postfixation (Uvarov *et al*, 2009) was used in KCC2a immunostainings for the earlier time points: E18, P3 and P12.

New polyclonal antibodies against the KCC2b isoform were generated in chicken, and specificity of the antibodies was tested by western blot (Study II, Fig 2). In addition, a previously characterized KCC2b-specific antiserum (Uvarov *et al*, 2009, Hubner *et al*, 2001) was used to stain some mouse brain sections, and produced an identical labeling as the new chicken KCC2b antibodies.

Our results showed that in general KCC2b expression increased postnatally especially in cortical regions, and expression was rather uniform in adult mouse brain, as has been reported previously. KCC2a immunoreactivity did not increase in cortical regions during development. Expression of KCC2a was found in several noncortical areas in mature mouse brain including the basal forebrain, hypothalamus, brainstem, and spinal cord. A summary of the postnatal KCC2a expression pattern is presented in Fig. 7.

4.2.1 Telencephalon

Previously it has been shown in *in situ* hybridization experiments, that KCC2 mRNA (both isoforms) can be seen in rodents in some regions of telencephalon before birth: such as the olfactory bulb, basal ganglia, amygdala and piriform cortex (Li *et al*, 2002, Wang *et al*, 2002, Stein *et al*, 2004). During postnatal development, expression gradually spreads into the neocortex and hippocampus, and adult levels are reached at P15 in mice (Wang *et al*, 2002, Stein *et al*, 2004, Rivera *et al*, 1999).

In immunohistochemistry, the overall pattern of KCC2a and KCC2b immunoreactivity in E18 mice was very similar (Uvarov *et al*, 2009): both isoforms were detected in the basal forebrain, while immunorectivity in cortical regions was undetectable.

In study II, KCC2b immunoreactivity was widely seen in adult mouse neocortex and hipppocampus, while KCC2a immunoreactivity was very low in these regions in adult mouse (Study II; Figs 3 and 4). KCC2a immunoreactivity was mainly restricted to parts of the basal forebrain. However, while the distribution of KCC2b signal was strong in most areas of the basal forebrain in adult mouse, the KCC2a isoform was strongly expressed mainly in the globus pallidum and parts of the ventral pallidum but expressed was not seen in the caudate putamen. KCC2a signal was also low or absent the olfactory cortex (piriform area, olfactory tubercle).

Although only very weak KCC2a labeling is seen in adult mouse cortex and hippocampus in general (Study II, Fig 3), we noted that clear KCC2a immunoreactivity is present in the subiculum of the hippocampal formation (Study III, Fig 5). Otherwise, the KCC2a signal in the hippocampus proper was very low or absent.

4.2.2 Diencephalon

In *in situ* hybridization experiments, KCC2 mRNA (both isoforms) have been detected in the hypothalamus and thalamus already at E12 when the diencephalon begins to form (Li *et al*, 2002, Wang *et al*, 2002, Stein *et al*, 2004, Mikawa *et al*, 2002). In thalamus, KCC2 mRNA is first present in the dorsolateral nuclei where neuronal differentiation originates, while the dorsomedial parts do not express KCC2 until E18.5 (Li *et al*, 2002, Wang *et al*, 2002). It has also been noted, that KCC2 mRNA expression is downregulated after birth in the ventral posterior thalamic nucleus (Wang *et al*, 2002).

In immunohistochemistry using the KCC2pan antibody in adult rat, immunostaining has been observed in all thalamic nuclei except the majority of the reticular nucleus (Bartho *et al*, 2004). Also, various relay nuclei expressed different levels of KCC2: Immunoreactivity was the strongest in primary sensory relay nuclei (first order nuclei), in particular, the somatosensory ventral posterolateral and ventral posteromedial nuclei. In diencepalon, prominent KCC2pan immunoreactivity was also observed in zona incerta, ventral lateral geniculate nucleus, and anterior pretectal nucleus.

In our study, both isoforms showed a rather similar pattern of strong labeling in mouse hypothalamus at E18.5, P3 and adult time points (Study II; Fig 5). At E18.5, the signal in hypothalamus was clearly stronger than in thalamus. At P3, KCC2a signal was the strongest in the region of hypothalamus, whereas KCC2b signal was clearly seen in the hypothalamus and thalamus and some signal was also detectable in cortical regions. In adult hypothalamus, KCC2a and KCC2b immunoreactivities were intense and seen evenly throughout hypothalamus.

In thalamus both isoforms showed a rather similar pattern of moderate or low labeling at E18.5 (Study III; Fig 5). All parts of the P3 thalamus were clearly positive for KCC2b, whereas KCC2a labeling was low or absent in most parts of the thalamus proper. In adult mouse, KCC2b was widely expressed in most parts of the thalamus. Most parts of the thalamus in adult mouse lacked KCC2a signal, immunoreactivity was seen only in the dorsomedial part and midline thalamic nuclei and in the ventral lateral geniculate nucleus.

It has previously been reported that KCC2 mRNA expression in the habenular complex is very weak (Kanaka *et al*, 2001): The lateral habenular nucleus contained weakly labeled neurons, but no KCC2 mRNA signals were detected in the medial habenular nucleus. In another study, the inferior part of the medial habenula was demonstrated to lack KCC2 protein expression (Kim and Chung, 2007). In our study, the large inferior part of the medial habenula also lacked KCC2 expression, but both isoforms were clearly detected in the superior part of the medial habenula (Study III, Fig 5). Labeling for both KCC2a and KCC2b was concentrated in the large dendritic glomeruli. Both KCC2 isoforms were clearly expressed in the lateral habenula.

4.2.3 Brainstem

In *in situ* hybridization experiments, KCC2 transcripts (both isoforms) have been first detected in the immature brainstem by E10.5 in mice (Stein *et al*, 2004). In adult rat brainstem, strong KCC2 mRNA expression was observed in most parts of midbrain, pons and medulla (Kanaka *et al*, 2001). KCC2 mRNA was however not detected in the mesencephalic trigeminal nucleus (Kanaka *et al*, 2001).

In our study, both KCC2 isoforms were widely and abundantly present in the large part of the brainstem (midbrain, pons and medulla) in P3, P12 and mature mouse (Study III; Fig 3 and 6). However, KCC2a appeared to be selectively absent in the main auditory pathway neurons: In the midbrain, KCC2a immunoreactivity was relatively abundant in the superior colliculus but low or absent in the inferior colliculus. In the medulla, KCC2a immunoreactivity was absent in a central part of the ventral cochlear nucleus and the superior olivary complex (SOC). The lack of KCC2a immunoreactivity in SOC was observed already in P3 mice.

The SOC consists of several nuclei, the main ones are the medial nucleus of the trapezoid body (MNTB), the medial superior olive (MSO), the lateral superior olive (LSO), and the superior paraolivary nucleus (SPN). All these nuclei appeared to lack KCC2a immunoreactivity, while KCC2b was clearly expressed in developing and adult mouse SOC nuclei (Study II, Fig 6).

Previously it has been reported that KCC2pan immunoreactivity in the SOC nuclei is present already at P0 without a substantial increase during maturation (Lohrke *et al*, 2005). It was also observed, that KCC2 protein expression precedes the functional maturation of the

transporter in neurons of all SOC nuclei. The development from depolarizing to hyperpolarizing glycine action in SOC nuclei is completed by the onset of hearing (around P12), but plasma membrane-associated KCC2 immunoreactivity was found already at times when glycine was still depolarizing (Lohrke *et al*, 2005). This implies that the mere presence of KCC2 protein is not sufficient for hyperpolarizing glycine action in the various SOC nuclei.

The functional activation of KCC2 has been studied particularly in the LSO, where the shift from depolarizing to hyperpolarizing glycine responses occurred at P4–5 (Lohrke *et al*, 2005). KCC2b immunoreactivity was abundant present in the plasma membrane, or in close proximity to it, in LSO neurons already at P0, when Cl^- extrusion is not yet effective and glycine is depolarizing. This implied the presence of transport-inactive KCC2 protein during early development and that KCC2 transport activity in developing LSO neurons is not regulated via an intracellular retention mechanism (Blaesse *et al*, 2006).

In LSO neurons from KCC2b-deficient mice, the intracellular Cl^- concentration was affected in P12 neurons but not in immature P3 neurons, when compared to wild-type neurons. As the KCC2a isoform, according to our study, is not expressed in LSO neurons, the KCC2b isoform is thus responsible for the developmental increase in neuronal Cl^- extrusion in P12 LSO neurons as seen in these mice. In P3 LSO neurons KCC2b is thus expressed at a high level in a functionally inactive form, and the neurons generate depolarizing GABAA and glycine responses (Balakrishnan *et al*, 2003).

The reason for (and consequences of) the lack of KCC2a in auditory brainstem neurons is not known. However, efficient inhibition is very important in auditory neurons and particularly in SOC in the process of sound localization. The MNTB nucleus contains principal glycinergic neurons that provide a strong inhibitory input onto the other SOC nuclei (MSO, LSO, and SPN). The main task of this inhibition by MNTB is to convert the excitatory input from the contralateral ear into a well-timed inhibitory input onto neurons of other SOC nuclei (Johnston *et al*, 2010, Nothwang, 2016).

In the SPN, a synaptic target of the MNTB, plasticity of inhibition has been observed to take place via modulation of the KCC2 level (Yassin *et al*, 2014). The SPN neurons were able to modify the Cl^- equilibrium potential and inhibitory strength of glycine via modulation of the KCC2 expression. Nitric oxide was shown to act postsynaptically through a cGMP dependent pathway to suppress KCC2 protein expression in SPN neurons and cause a depolarizing shift in reversal potential (Yassin *et al*, 2014).

4.2.4 Cerebellum

In mouse cerebellum, Purkinje cells already express KCC2 at E15.5 when they start to differentiate. At P3, KCC2 mRNA can also be detected in granular cells in mice, and expression increases with maturation (Stein *et al*, 2004). Cerebellar neurons continue to

exhibit marked up-regulation of KCC2 mRNA during postnatal development, and the adult pattern is reached at P21 in mouse and rat (Li *et al*, 2002, Mikawa *et al*, 2002).

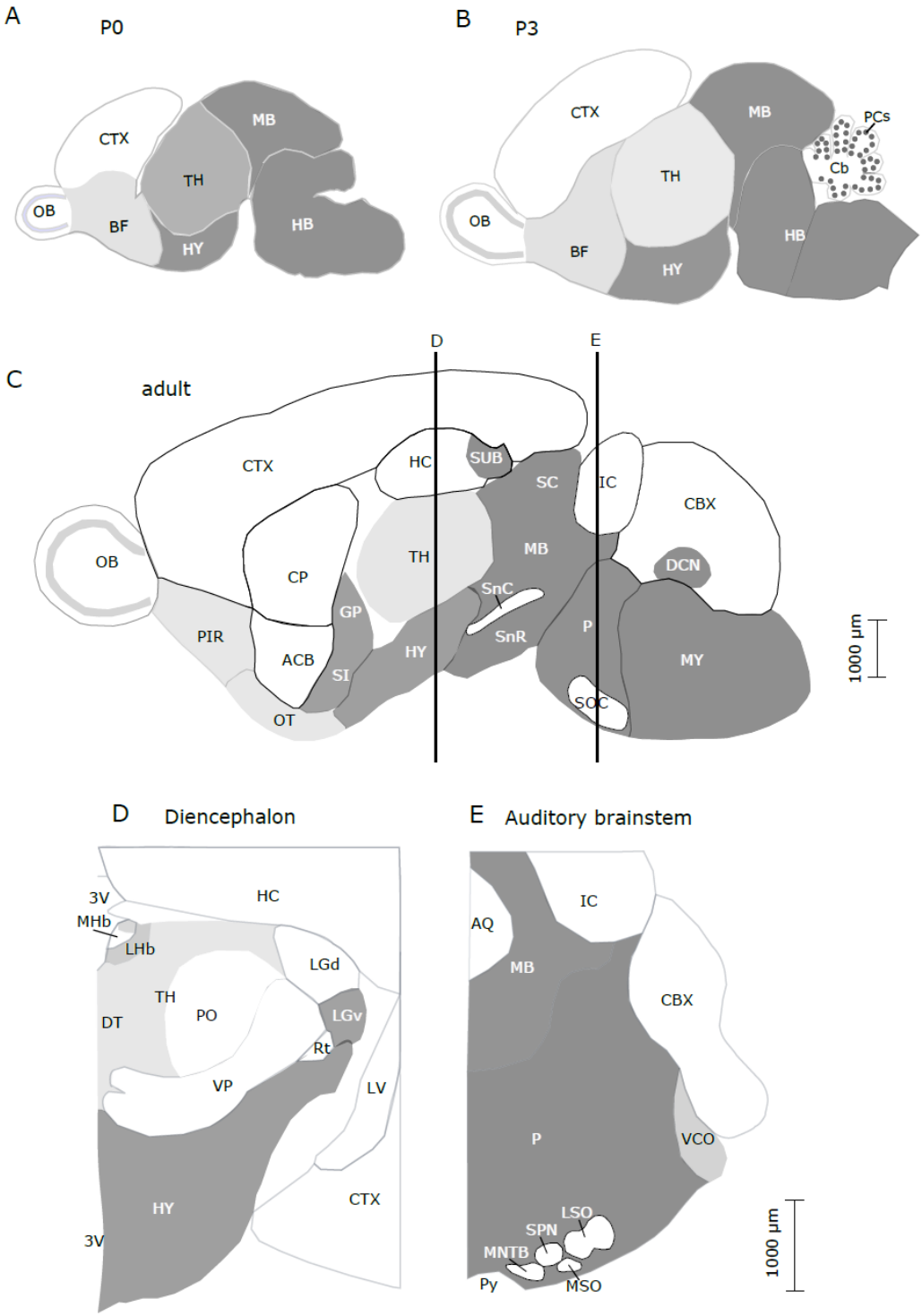
In previous reports, KCC2pan immunoreactivity was observed in mouse Purkinje cells at E17 and the signal increased with maturation and at P10 dendrites and cell bodies of Purkinje cells were clearly labeled (Takayama and Inoue, 2006). In Purkinje cells, KCC2 shifts from a diffuse cytoplasmic pattern to a predominant cell surface distribution between P7 to P15. Simultaneously KCC2 is preferentially recruited to climbing fiber synapses, augmenting the inhibitory GABAergic actions by incoming basket cell axons (Kawakita *et al*, 2013). In the internal granule cell layer at P5, KCC2pan immunoreactivity was observed after formation of first excitatory synapses with mossy fiber terminals, and at P10 KCC2pan positive synaptic glomeruli were clearly discernible (Takayama and Inoue, 2006). At P0 and P3, KCC2pan immunoreactivity was observed in the developing molecular layer and at P10 and in adult, dendrites and cell bodies of stellate and basket cells were clearly labeled (Takayama and Inoue, 2006).

KCC2 expression is rather uniform in the mature cerebellum. The granule cell layer of the adult mouse cerebellum has been shown to exhibit the most intense KCC2pan staining, and particularly intense immunostaining was observed in glomeruli within the granule cell layer (Williams *et al*, 1999). KCC2b immunoreactivity was noted to be stronger in ventrocaudal than in dorsal parts of the cerebellar cortex (Blaesse *et al*, 2006).

We observed both KCC2a and KCC2b immunoreactivity in Purkinje cells at P3 and P7, but at P12 and adult, only KCC2b immunoreactivity was seen in Purkinje cells (Study II; Fig 6). It seems that KCC2a was expressed only transiently in the Purkinje cells. KCC2b immunoreactivity was seen both in the Purkinje cell somata and in dendrites at all developmental time points analyzed (E18, P3, P7, P12, and adult) and was associated with the somatic and dendritic plasma membrane in adult. In the granule cell layer, in granule cells and their dendritic glomeruli, KCC2b was clearly seen at P12 and was further increased with maturation. KCC2a signal was not detectable in the cerebellar granule cells at any age studied (P3, P12, adult). In adult mouse cerebellum, KCC2b immunoreactivity was rather uniform, but stronger in ventrocaudal parts, as reported previously (Blaesse *et al*, 2006).

Although KCC2a immunoreactivity was not observed in the cerebellar cortex (except in the Purkinje cells at P3 and P7), KCC2a was clearly expressed in deep cerebellar nuclei (interposed nucleus, fastigal nucleus and dentate nucleus) of P12 and adult mice. Also, KCC2b was strongly expressed in cerebellar nuclei in P12 and adult mice.

Fig.7 Expression pattern of KCC2a protein in postnatal mouse brain
The KCC2a expression pattern is represented in schematic drawings of mouse brain sections at three postnatal time points. Expression is indicated by gray colored areas as observed in the immunostainings: Lighter gray color corresponds to a low signal intensity and darker gray color to a high signal intensity.
A) At birth, KCC2a is detected in hindbrain, midbrain and hypothalamus. Labeling is moderate/low in thalamus, basal forebrain and, olfactory bulb. KCC2a is undetectable in cortical regions. On the whole, the pattern of KCC2a labeling is identical to the pattern of KCC2b expression at this time point.
B) At P3, KCC2a is also detected in Purkinje cells of the cerebellum, similar to KCC2b. In the thalamus, KCC2a signal has decreased, in contrast to KCC2b that is already strongly expressed in thalamus at P3. KCC2a is not detected in cortical regions, whereas weak KCC2b labeling is already detectable in cortical regions in the P3 mouse.
C) In the adult mouse, KCC2a labeling is very low in cortical regions (particularly in the neocortex) where KCC2b is now widely expressed. KCC2a is, however, detected in subiculum, although signal in the hippocampus proper is very low or absent. KCC2a is also low/absent in layers of the olfactory cortex including the piriform area and the olfactory tubercle. In the adult striatum, KCC2a is detected in the globus pallidum and parts of the ventral pallidum, but not in the caudate putamen. KCC2a signal is absent in most parts of the adult thalamus (thus KCC2a seems to disappear from thalamus by adulthood). In cerebellum KCC2a was detected in deep cerebellar nuclei, but the cerebellar cortex, including Purkinje cells, lacked KCC2a (thus KCC2a is apparently expressed in Purkinje cells only transiently, as signal is detected at P3 and P7 but not in P12 and adult). KCC2a labeling is abundant in brainstem, but some areas such as the superior olivary complex are negative.
D) A frontal section through adult diencephalon (level indicated in C). KCC2a signal is strong in hypothalamus but absent in most parts of thalamus except for dorsomedial and midline thalamic nuclei, as well as the ventral lateral geniculate nucleus of thalamus. KCC2a is detected in the lateral habenula and in the superior part of the medial habenula in the dendritic glomeruli.
E) A frontal section through adult pons (level indicated in C). KCC2a is widely present in adult brainstem, but signal is weak/absent in auditory system neurons, in particular in a central part of the ventral cochlear nucleus and in nuclei of the lateral superior olivary complex. KCC2a signal is also very low/absent in the inferior colliculus. (KCC2a labeling is also absent in the auditory medial geniculate nucleus of thalamus.)
Abbreviations: ACB, nucleus accumbens; AQ, aqueduct; BF, basal forebrain; CBX, cerebellar cortex; CP, caudate-putamen; CTX, cerebral cortex; DCN, deep cerebellar nucleus; DT, dorsal thalamus; GP, globus pallidus; HB, hindbrain; HC, hippocampus; HY hypothalamus; Hyp, hypothalamus; IC, inferior colliculus; LGd, dorsal lateral geniculate nucleus; LGv, ventral lateral geniculate nucleus; LHb, lateral habenula; LSO, lateral superior olive; LV, lateral ventricle; MB, midbrain; MHb, medial habenula; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; MY, medulla; OB, olfactory bulb; OT, olfactory tubercle; P, pons; PCs, Purkinje cells; PIR, piriform area; PO, posterior complex of the thalamus; Py, pyramidal tract; Rt, reticular nucleus of the thalamus; SC, superior colliculus; SI, substantia innominate; SNc, substantia nigra, compact part; SNr, substantia nigra, reticular part; SOC, superior olivary complex; SPN, superior periolivary nucleus; SUB, subiculum; TH, thalamus; 3V, third ventricle; VCO, ventral cochlear nucleus; VP, ventral posterior complex of the thalamus.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MNTB
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4.2.5 Spinal cord

KCC2 transcripts are detected in mice in the ventral horn of the spinal cord by E10.5 in *in situ* hybridization (Stein *et al*, 2004), and the expression spreads in a caudal-rostral fashion. In developing mouse motoneurons in the ventral horn of the spinal cord, KCC2 transcripts have been detected at E12.5 and in sensory neurons at later stages (Hubner *et al*, 2001). At E18.5, KCC2 transcripts were detectable throughout the spinal cord along the complete neuraxis (Hubner *et al*, 2001).

KCC2b immunostaining in the spinal cord developed along latero–medial and ventro–dorsal gradients (Stil *et al*, 2009). At P0, KCC2b immunolabeling was observed in the ventral horn and expression of KCC2b markedly increased in the whole spinal cord between P0 and P5. KCC2b immunoreactivity in the medial grey matter, where the central pattern generator - interneurons are located, and the most superficial layers of the dorsal horn was weak at P0 (Stil *et al*, 2009).

At E18.5 KCC2b immunostaining in the mouse ventral horn of spinal cord has been detected mainly in dendrites and in the adult, KCC2b was clearly localized at the plasma membrane of dendrites, enriched at the periphery of the postsynaptic specialization of inhibitory synapses (Hubner *et al*, 2001). KCC2b immunoreactivity was observed at the plasma membrane of somas and dendrites already at birth in motoneurons of mouse spinal cord. Dendrites throughout the neuropil and the white matter strongly expressed KCC2b at P5. Labeled neurites (dendrites) stretched out of the gray matter in into the lateral and ventral funiculi at this developmental time-point (Jean-Xavier *et al*, 2006).

We detected both KCC2 isoforms in the ventral part of mouse spinal cord already at E12 and in newborn, labeling for KCC2 isoforms showed a relatively similar even distribution in both dorsal and ventral parts (Study III, data not shown). This is in line with previous data demonstrating similar expression level of both isoforms in neonatal spinal cord (Uvarov *et al*, 2007, Uvarov *et al*, 2009). Abundant expression of both KCC2 isoforms in the large part of the developing and mature spinal cord was observed at P3 (Study III, Fig 7). Similarly, in adult mouse spinal cord, the KCC2a and KCC2b labeling showed a relatively similar and even distribution. We also observed KCC2a and KCC2b immunoreactivity in putative dendrites extending into the white matter in adult spinal cord.

4.3 SUBCELLULAR LOCALIZATION

4.3.1 Different surface expression

Electron microscopic studies have demonstrated that most KCC2pan and KCC2b immunoreactivity is found associated with the plasma membrane both in somatic and dendritic compartments in mature neurons (Gulyas *et al*, 2001, Baldi *et al*, 2010, Gulacsi *et al*,

2003, Bartho *et al*, 2004, Szabadics *et al*, 2006, Takayama and Inoue, 2006, Belenky *et al*, 2008, Hubner *et al*, 2001, Boulenguez *et al*, 2010, Blaesse *et al*, 2006).

The subcellular localization of KCC2a and KCC2b was studied in several areas of adult mouse CNS using double-staining with isoform-specific antibodies and confocal microscopy analysis (Study II; Fig 8). In PFA-fixed brain sections, KCC2b immunoreactivity was seen at the plasma membrane of the somas and proximal dendrites of virtually all CNS neurons, while KCC2a was not clearly detected at the surface. In study II we focused on two areas with high KCC2a expression in adult mouse: deep cerebellar nucleus and pons. In neurons of the deep cerebellar nucleus, KCC2b was clearly concentrated at the plasma membrane in the soma region, while KCC2a signal was not detectable at the surface of neuronal somas or in the region of proximal dendrites. Similar results were obtained in the region of the pons, where only the KCC2b isoform is seen at the surface of the soma and proximal dendrites.

KCC2a signal also co-localized with MAP2, as shown in a confocal microscopy image in a section through the pons double-stained with anti-KCC2a and anti-MAP2 (Study II; Fig 8). KCC2a thus is preferentially located in neuronal dendrites.

In study III we further analyzed KCC2a and KCC2b surface expression in PFA-fixed adult mouse brain, but focused on brain areas with medium level of KCC2a expression: subiculum and hypothalamus (Study III; Fig 5). Similar to study II we used double-staining with isoform-specific antibodies and confocal microscopy analysis. In subiculum, KCC2a labeling was clearly seen at the neuronal soma surface of neurons, similar to KCC2b labeling. In some hypothalamic neurons, KCC2a was also seen at the soma surface although not as clearly as the KCC2b signal. In both subiculum and hypothalamus, KCC2b labeling was clearly detected at soma surface and the area of proximal dendrites. For comparison, KCC2b but not KCC2a labeling was detected at the plasma membrane of neuronal somas in the medulla oblongata, as observed in pons and deep cerebellar nuclei in study II.

In study III we also showed that KCC2b immunoreactivity is localized at the plasma membrane of neurons in sections from PFA-fixed KCC2a-KO mouse brain as seen in wild-type. Confocal microscopic images of anti-KCC2b labeled neurons from the hypothalamus, subiculum, CA1 and medulla were presented (Study III; Suppl. Fig 1).

Moreover, we found that KCC2pan immunoreactivity can be seen at the surface in some neurons in PFA-fixed brain sections from PFA-fixed KCC2b-KO mouse (Woo et al., 2002) in the pons region. However, as in wild type, plasma membrane KCC2a immunoreactivity was not detected by the KCC2a antibody in KCC2b-deficient neurons (Study III; Suppl. Fig 5). Since the KCC2pan antibody recognizes the common C-terminus of KCC2 (Williams *et al*, 1999), and KCC2a isoform is the only expressed KCC2 isoform in these mice (Uvarov *et al*, 2007) the KCC2 pan immunoreactivity in these mice represents the KCC2a distribution. Thus, it seems that KCC2a can be detected at the surface of neurons in the pons when using the KCC2pan antibody, but not with the N-terminal KCC2a antibody.

The surface expression of endogenous KCC2 isoforms was also examined in two-week-old dissociated rat cortical cultures fixed with methanol (Study III; Fig 4). Immunostaining with the isoform specific antibodies revealed that KCC2b immunoreactivity was observed in all neurons at that time point, and in about half of them KCC2b signal was observed at the plasma membrane of the soma and proximal dendrites, while the cytoplasm was only weakly labeled. In contrast, KCC2a-immunoreactivity was detected exclusively in the intracellular compartments and it was not observed at the surface of somas or proximal dendrites in any of the neurons. Surprisingly, biotinylation assay in the same kind of neuronal cultures (two-week-old dissociated rat cortical cultures) revealed that the relative surface representation of KCC2a and KCC2b isoforms (monomers) was about 40 % of the total expression level for each of the isoforms (Study III; Fig4). The discrepancy between the immunostaining and biotinylation data also suggests that the N-terminal epitope of KCC2a may be masked at the plasma membrane.

The differential surface expression of endogenous KCC2 isoforms was also observed in cultured mouse hippocampal neurons fixed with methanol (Study III; Suppl. Fig 4). The hippocampal neurons were stained with KCC2a or KCC2pan antibodies at two different time-points: DIV5 and DIV19. KCC2a and KCC2pan immunoreactivity was present in cell bodies and dendritic shafts already at DIV5 but immunoreactivity was predominantly intracellular with both antibodies. In DIV19 neurons the KCC2a labeling was largely similar as in DIV5, only a weak KCC2a signal could be seen at the soma surface in some neurons. KCC2pan-immunoreactivity was clearly and predominantly detected at the neuronal surface in DIV19 neurons. Thus, in the more mature hippocampal neurons (DIV19) and cortical neurons (DIV14), a similar difference in localization of endogenous KCC2 isoforms is observed.

To conclude, our results indicate that KCC2a immunoreactivity (when using the N-terminal isoform specific antibody) is not detected at or near the plasma membrane of somas and proximal dendrites of neurons in most brain regions. The immunostaining data from cultured neurons is also in agreement with results from immunostainings of brain sections. The resolution of confocal microscopy is not enough in order to determine actual vs. near membrane expression, but the results clearly show the different distribution of KCC2a immunoreactivity compared to KCC2b when using isoform specific antibodies: KCC2a immunoreactivity in soma and proximal dendrites is concentrated in intracellular compartments but it is not seen at or near the plasma membrane. With confocal microscopy it is also not possible to analyze the intracellular vs surface expression in the thin distal dendrites.

It is possible that the intracellular retention or targeting to the cell surface might be different for the KCC2 isoforms as the unique N-terminal part of KCC2a may contain targeting signals different from those of KCC2b. Indeed, truncation of the N terminus of KCC2b has been shown to affect the surface expression of the protein (Friedel *et al*, 2017). However, when using the C-terminal KCC2pan antibody, KCC2a can be detected at the surface of neurons in the pons in KCC2b-deficient mice, rather suggesting that the N-terminal epitope of KCC2a at the surface is masked. Results from biotinylation assay also support the hypothesis

of the masked N-terminal epitope, as both KCC2 isoforms are similarly present at the plasma membrane of two-week-old cultured cortical neurons in contrast to what was observed in immunocytochemistry.

The N-terminal epitope of KCC2a and/or KCC2b may be differentially masked, for example by a protein that binds to the N-terminal epitope of KCC2a at the plasma membrane. A possible candidate for the KCC2a epitope masking is SPAK since the binding motif RFx(V/I) for SPAK is located in the unique N-terminal part of the KCC2a, near the anti-KCC2a epitope (Uvarov *et al*, 2007). The heterogeneous expression pattern of SPAK protein (Ushiro *et al*, 1998, Johnston *et al*, 2000, Piechotta *et al*, 2003) in the mouse brain could explain why KCC2a immunoreactivity is detected nearby neuronal surface in some but not other brain regions. For example, SPAK mRNA expression pattern in adult mouse brain is low in subiculum and hypothalamus, while much stronger in deep cerebellar nucleus or medulla (Allen Brain Atlas) (Study III; Suppl. Fig. 6).

4.3.2 Different subcellular targeting

In immunostaining of adult mouse brain sections, KCC2a is preferentially located in neuronal dendrites (co-localized with MAP2) (Study II; Fig. 8), and KCC2a and KCC2b immunoreactivity are overlapping in many parts of dendrites. Previously a similar cellular distribution of KCC2 isoforms was observed in immunohistochemistry in E18 mouse brain where most neurons in non-cortical brain structures were positive for both KCC2a and KCC2b and most positive neurons were observed to co-express KCC2a and KCC2b (Uvarov *et al*, 2009). As KCC2a and KCC2b have also been shown to form heterodimers *in vivo* (Uvarov *et al*, 2009), the co-localization could represent such subdomains were heterodimers are present.

However, in the dendrites, KCC2a localization seems to be at least partially different from that of KCC2b as the signals are not completely overlapping in KCC2a-KCC2b double-stainings in deep cerebellar nucleus and pons (Study II; Fig. 8), as well as in subiculum, hypothalamus and medulla (Study III; Fig 5). It appears that KCC2a signal could be more localized to distal dendrites as compared to KCC2b signal that is clearly seen in soma and proximal dendrites. Also in a section of adult mouse lumbar spinal cord, double-labeled with anti-KCC2a and anti-KCC2b, the distribution of KCC2 isoforms seems to be somewhat different in the putative dendritic branches projecting into the white matter (Study II; Fig. 7).

In electron microscopic studies, KCC2pan immunoreactivity is usually evenly distributed along the somato-dendritic axis of neurons and is seen along the membrane of dendrites with different diameters (Takayama and Inoue, 2006, Bartho *et al*, 2004, Baldi *et al*, 2010). However, in CA1 pyramidal neurons the dendritic distribution of KCC2 was somewhat heterogenous (Baldi *et al*, 2010). In OFF bipolar cells and starburst cells of the retina, KCC2 is confined in distal dendrites (Vardi *et al*, 2000, Gavrikov *et al*, 2006). KCC2b isoform

immunoreactivity is also concentrated at the plasma membrane of somas and dendrites in spinal motoneurons (Boulenguez *et al*, 2010, Hubner *et al*, 2001, Stil *et al*, 2011) and in brainstem auditory neurons (Blaesse *et al*, 2006) and enriched near inhibitory and excitatory synapses (Blaesse *et al*, 2006, Hubner *et al*, 2001).

The partly different dendric localization of KCC2 isoforms could reflect different subcellular targeting of the isoforms to different parts of the neuron. For example, work on NKCC1 in epithelial cells suggests that alternative splicing can play a major role in differential subcellular targeting (Carmosino *et al*, 2008). However, mechanisms that underlie the differential subcellular targeting of distinct CCCs to different somato-dendritic compartments are not known. Several mechanisms could be involved including oligomerization, and protein-protein interactions for example with scaffolding molecules. The mechanisms of KCC2 clustering to various subdomains may also involve cholesterol-enriched lipid rafts (Watanabe *et al*, 2009).

However, as discussed in the previous section (4.3.1), the lack of KCC2a/KCC2b co-localization could also result from masking of the KCC2a epitope in some dendritic locations, for example in proximal dendrites and soma. Alternatively, the N-terminal epitope of KCC2b could also be masked in some subcellular locations. A hypothetical model of KCC2 isoforms and their targeting in a mature neuron is shown in Fig. 8, also taking into account the possible effect of masking of the KCC2a N-terminus.

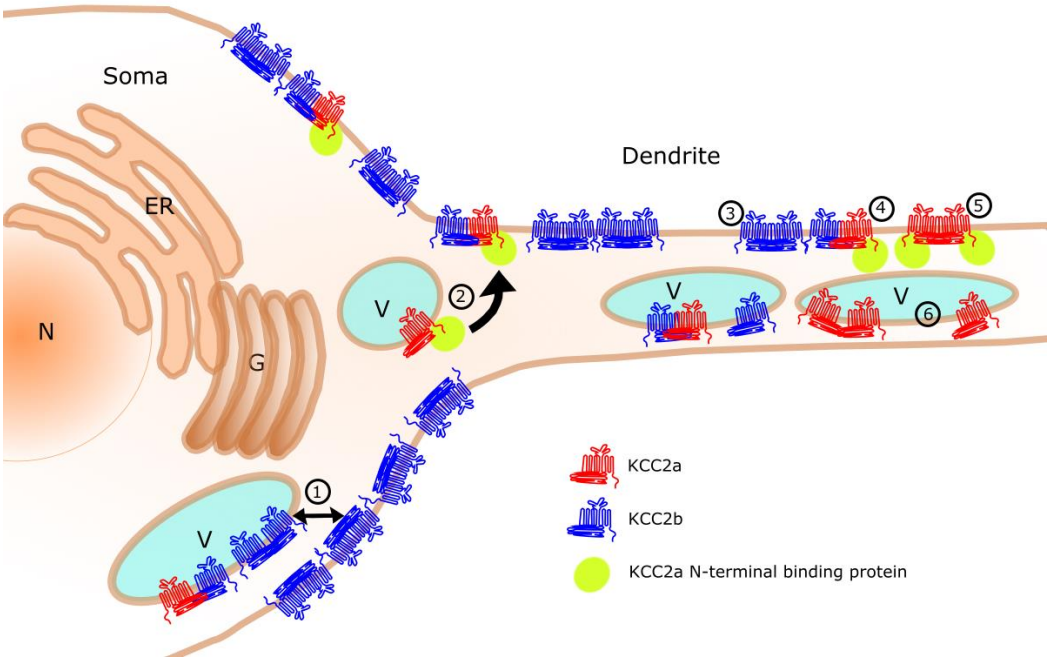

Fig. 8 Hypothetical scheme on the targeting of KCC2a and KCC2b in a mature neuron.
A model of a mature neuron, expressing KCC2a and KCC2b isoforms (KCC2b being the more abundant isoform). KCC2a and KCC2b are both equally present in the plasma membrane according to our biotinylation assay, while a fraction of KCC2 molecules are in intracellular compartments, presumably in vesicles (V) near the plasma membrane. KCC2 molecules are recycled between the plasma membrane and intracellular compartments (1). The unique N-terminus of the KCC2a isoform might require additional modifications (protein-protein interactions, phosphorylation) in order to be transported to the plasma membrane. In this model, a protein binds the KCC2a N-terminus before it is transported to the membrane (2) and this masks the KCC2a N-terminus at the plasma membrane. KCC2 has been suggested to function as oligomers in the plasma membrane (presumably as dimers), but details about how or where they form is not known. In a mature neuron, KCC2b-KCC2b homodimers/oligomers (3) would probably be the predominant form, while a fraction would represent KCC2a-KCC2b heterodimers (4), and even smaller fraction could be KCC2a-KCC2a homodimers (5). The regulation of these different oligomers might differ due to different interacting partners or phosphorylation etc. Immunostainings show that KCC2a and KCC2b labeling are often co-localized and KCC2a and KCC2b are thus probably often localized to the same microdomains (for example membrane rafts). However, we also observed non-overlapping KCC2a and KCC2b immunoreactivity, suggesting that the isoforms might occasionally localize in different microdomains. In this model, KCC2a is shown to aggregate in some vesicles that do not contain KCC2b in the distal dendrite (6). N, nucleus; ER, endoplasmic reticulum; G, Golgi apparatus; V, vesicle

4.4 FUNCTION AND REGULATION

4.4.1 KCC2a functionality

Previous studies suggested involvement of the N-terminus of the K-Cl cotransporters into regulation of the transport activity (Casula *et al*, 2001, Shen *et al*, 2003, Li *et al*, 2007, Horn *et al*, 2010, Fiumelli *et al*, 2013). It has also been shown previously that both KCC2 isoforms demonstrate similar levels of chloride transport activity in rubidium-flux assays when overexpressed in HEK293 cells (Uvarov *et al*, 2007), showing that KCC2a protein can be transported to the cell surface in hEK293 cells and function as a chloride transporter. However, in dissociated cortical neurons derived from KCC2b-knockout embryos, where KCC2a is the only expressed KCC2 isoform, no significant decrease in intracellular chloride concentration $[Cl^-]_i$ after 3 weeks in culture was revealed, in contrast to the significant decrease of $[Cl^-]_i$ in wild-type neurons (Zhu *et al*, 2005).

In study III we set out to study the functionality of KCC2a in cultured neurons as the model system. In previous reports (Ludwig *et al*, 2003, Fiumelli *et al*, 2005, Zhu *et al*, 2005, Khirug *et al*, 2005, Leonzino *et al*, 2016), the total KCC2 expression increased ~5-fold during the first week and ~3-fold during the second week in culture. Developmental upregulation of the KCC2 protein expression has been observed in dissociated mouse cortical cultures by ICC (Zhu *et al*, 2005) and in mouse hippocampal cultures by Western blot (Fiumelli *et al*, 2005),.

We started by studying the expression of endogenous KCC2 isoforms in cortical cultures at the level of protein and mRNA at DIV2, DIV8 and DIV14 (Study III; Fig. 1). To assess the proportion of the KCC2a isoform to the total KCC2 (KCC2a+KCC2b) protein expression in maturing cortical neurons, we used the previously developed method of running of KCC2a protein standards on the same SDS-PAGE alongside with experimental protein lysates (Uvarov *et al*, 2009). Even though KCC2a levels also increased during the two weeks in culture, the upregulation was not so pronounced (~3.5-fold for the 1st week, and ~2-fold for the 2nd week) compared to the total KCC2 levels. Proportion of KCC2a to total KCC2 protein expression decreased gradually with maturation of dissociated cortical cultures. The relative proportion of KCC2a to the total KCC2 protein expression was ~40 % in DIV2, ~30 % in DIV8, and ~20 % in DIV14 dissociated cortical cultures. Quantitative RT-PCR was used to measure KCC2a and KCC2 total mRNA levels in DIV2, DIV8 and DIV14 dissociated cortical cultures, and the developmental profile of mRNA data was in a good agreement with the protein data.

We also studied the efficacy of chloride extrusion mediated by overexpressed KCC2a in dissociated hippocampal cultures using electrophysiology (Study III; Fig. 2). One-week-old neuronal cultures derived from mouse embryos deficient for both KCC2 isoforms were transfected with KCC2a and analyzed two days after the transfection. Whole-cell patch-clamp recordings from soma under a constant Cl^- load via a somatic patch pipette were performed and the somatodendritic gradient of reversal potential E_{GABA} was measured (Khirug *et al*, 2005). The imposed E_{GABA} gradient was significantly larger in the KCC2a-transfected neurons compared to neurons transfected with the empty vector. Thus, exogenous KCC2a promotes active chloride extrusion and increases E_{GABA} gradient in cultured neurons.

In addition, we studied the efficacy of chloride extrusion mediated by overexpressed KCC2a in dissociated rat cortical cultures using calcium imaging (Study III; Fig 3). Wild-type neuronal cultures were used in this experiment, but calcium transients were recorded at DIV6 when endogenous KCC2 expression is still low (Ludwig *et al*, 2003, Khirug *et al*, 2005, Uvarov *et al*, 2006, Fiumelli *et al*, 2005) and $GABA_A$ responses are predominantly depolarizing (Chudotvorova *et al*, 2005, Khirug *et al*, 2005). The cultures were transfected with a plasmid expressing either one of the KCC2 isoforms or just an empty vector as a control. Calcium responses were recorded after application of $GABA_A$ receptor agonist muscimol to the neuronal cultures. Depolarizing $GABA_A$ responses can directly activate voltage-gated calcium channels, and thus result in increased intracellular calcium levels that can be recorded using Fura-2 AM calcium imaging protocol. We asked whether exogenous KCC2a overexpression in cultured neurons could also induce a shift in responses of $GABA_A$ receptors from

depolarizing to hyperpolarizing. Overexpression of either KCC2a or KCC2b isoform caused a dramatic decrease in the fraction of responding neurons (~15 %) compared to in neurons transfected with the empty vector (above 60%). There was no significant difference between KCC2a or KCC2b isoforms in this experiment, thus the KCC2a isoform is active in cultured neurons and can reduce the intracellular chloride concentration to the level sufficient for attenuating the depolarizing responses of the $GABA_A$ receptors.

In conclusion, our results show that overexpressed KCC2a isoform can decrease the intracellular chloride concentration in cultured neurons. However, data from cultured neurons from KCC2b-deficient mice could indicate that the KCC2a isoform might not be functional *in vivo* (Zhu *et al*, 2005). In that study, the KCC2b isoform clearly mediates the developmental decrease in intracellular Cl^- and is responsible for the developmental shift from depolarizing to hyperpolarizing GABAergic responses. On the other hand, our results also demonstrate that the KCC2a isoform constitutes only ~20 % (or less) of the total KCC2 expression in the mature cortical cultures and this could explain why $[Cl^-]_i$ remains unchanged in the cultured KCC2b-deficient cortical neurons.

4.4.2 Regulation by SPAK

The 40-amino-acid long N-terminus unique for KCC2a contains the binding motif for the SPAK kinase (Uvarov *et al*, 2007). Binding of SPAK to the KCC2 isoforms was studied in co-immunoprecipitation experiments in HEK293 cells (Study III; Fig. 6). In co-immunoprecipitation with the KCC2pan antibody from HEK293 lysates, SPAK interaction with KCC2a is substantially stronger than that with KCC2b. The co-immunoprecipitation assay was also performed using anti-HA antibody to precipitate protein complexes from the same HEK293 lysates. Precipitation with the anti-HA antibody was not as effective as with the KCC2pan antibody, yet detectable HA-SPAK enrichment was observed.

The functional ^{86}Rb assay in HEK293 cells was used to study regulation of the KCC2a and KCC2b transport activities by SPAK (Study III; Fig 7). HEK293 cells are a widely used mammalian expression system for CCC transporters (Moore-Hoon and Turner, 2000, Simard *et al*, 2004, Wenz *et al*, 2009), and have been extensively used for studying SPAK-mediated regulation of CCCs. HEK293 cells express endogenously KCC1, KCC4, and NKCC1 proteins (Xu *et al*, 1994, Simard *et al*, 2007), but neither KCC2a nor KCC2b (Payne *et al*, 1996, Williams *et al*, 1999, Uvarov *et al*, 2009). SPAK and OSR1 kinases, as well as their upstream kinase WNK1, are expressed endogenously in HEK293 cells (Vitari *et al*, 2006, Rinehart *et al*, 2009).

In our experiments, HEK293 cells were transiently transfected with constructs encoding either KCC2a or KCC2b isoforms. Rubidium assay was performed to measure the influx mode of the KCC2 transport activity (Payne, 1997, Uvarov *et al*, 2007). The $^{86}Rb^+$ flux was measured with and without furosemide (2 mM), an inhibitor of KCC cotransporters, to determine the furosemide-sensitive component of the K-Cl transport activity. Bumetanide

(10 μM), a potent inhibitor of NKCC1 protein (Xu *et al*, 1994), was added into all flux solutions to exclude NKCC1 impact on ^{86}Rb uptake. SPAK overexpression decreased the transport activity of KCC2a (by ~20 %) but not that of KCC2b. Since our experiment was performed in isotonic solution and without adding WNK, the activity of overexpressed SPAK was probably relatively low and this may be the reason for the relatively mild decrease in the transport activity of KCC2a. Regarding KCC2b isoform, it has been shown previously that SPAK overexpression in oocytes inhibited KCC2b transport activity in hypotonic/low chloride conditions even though KCC2b isoform lacks SPAK-binding motifs (Gagnon *et al*, 2006). However, KCC2b inhibition in isotonic conditions after SPAK overexpression in the absence of WNKs was not observed, similar to our experiment.

To test whether KCC2a and KCC2b isoforms are regulated by endogenous SPAK/OSR1 in HEK293 cells, we overexpressed the dominant negative SPAK (DNSPAK) (Dowd and Forbush, 2003) (study III; Fig. 7). This DNSPAK contains a single amino acid substitution (K101R) within the catalytic domain that impairs its kinase activity. DNSPAK can bind proteins at the same sites as SPAK, but cannot phosphorylate them. DNSPAK overexpression strongly enhanced the furosemide-sensitive ^{86}Rb uptake mediated by KCC2a (3.2-fold) and KCC2b (2.6-fold) relative to the cells transfected with KCC2a or KCC2b constructs only. Increase in the transport activity of KCC2b has been observed previously after overexpression of DNSPAK in Xenopus oocytes (Gagnon *et al*, 2006).

The functional ^{86}Rb assay was performed by substituting the K^+ by $^{86}Rb^+$ in the extracellular solution and the influx mode of the KCC2 transport activity was measured (Payne, 1997, Uvarov *et al*, 2007). When no SPAK is overexpressed endogenous SPAK/OSR1 in HEK293 cells are presumably active and inhibit the function of both KCC2a and KCC2b via phosphorylation. Endogenous WNKs in HEK293 cells may also function to inhibit the activity of KCC2 in a SPAK-independent manner, for example by inhibition of protein phosphatases. Endogenous SPAK/OSR1 may also regulate the function of other endogenous KCCs (KCC1, KCC3, KCC4) that are present in HEK293 cells. When SPAK is overexpressed, a relatively mild decrease in the transport activity of KCC2a was detected but not in that of KCC2b. The overexpressed SPAK probably binds KCC2a and is thus able to increases the phosphorylation status of KCC2a. When DNSPAK is overexpressed, it presumably binds KCC2a at the SPAK-binding site and prevents binding and phosphorylation by endogenous SPAK/OSR1. Since DNSPAK overexpression strongly enhanced the furosemide-sensitive ^{86}Rb uptake mediated by both KCC2a and KCC2b it implies that DNSPAK probably inhibits the activation (phosphorylation) mediated by endogenous SPAK/OSR1 in HEK293 cells, and KCCs are dephosphorylated. Again, the mechanism of DNSPAK is not clear regarding KCC2b, but DNSPAK could for example inhibit the repression of WNKs on protein phosphatases by binding endogenous WNKs. In Fig. 9 a hypothetical scheme on regulation of KCC2a and KCC2b by SPAK is shown.

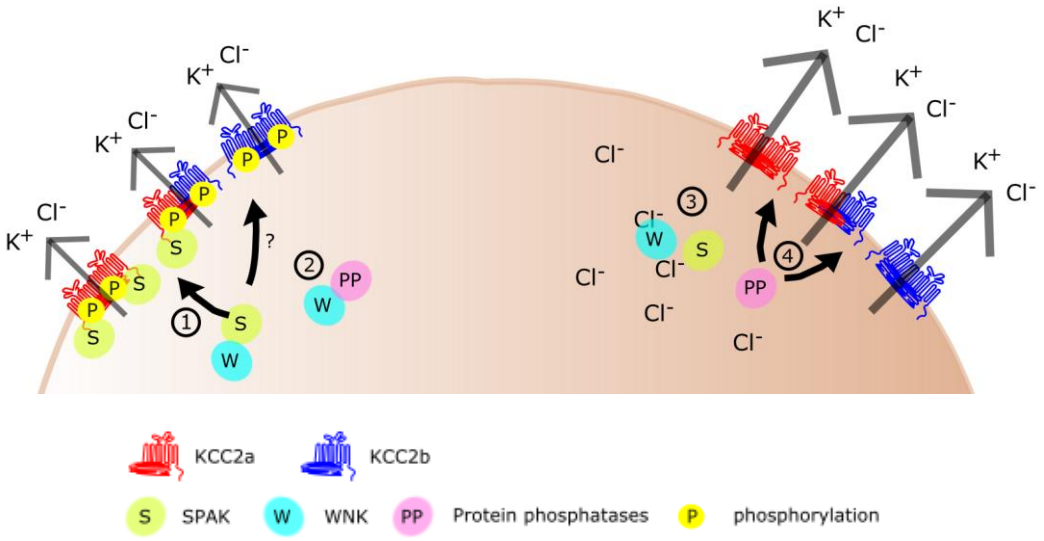

Fig. 9. Hypothetical scheme on the regulation of KCC2a and KCC2b by SPAK. KCC2 on the plasma membrane probably exists as KCC2b-KCC2b homodimers, KCC2a-KCC2a homodimers and KCC2a-KCC2b heterodimers. KCC2b homodimers would predominate in a mature neuron. Under normal physiological conditions (1), WNK is active and activates SPAK that binds to the SPAK binding site of KCC2a and thus is able to phosphorylate KCC2a in KCC2a-KCC2a homodimers and both KCC2a and KCC2b in KCC2a-KCC2b heterodimers. However, KCC2b molecules in KCC2b-KCC2b homodimers are also phosphorylated by the WNK-SPAK pathway, but since SPAK does not seem to bind KCC2b in HEK cells, the mechanism is not clear. Simultaneously, protein phosphatases are inactivated by WNK (2) and this helps to maintain the phosphorylation state of KCC2 molecules. WNKs may function to inhibit the activity of KCC2b in a SPAK-independent manner via inhibition of protein phosphatases. When the KCC2 molecules are phosphorylated, as in normal physiological conditions, the ion-transport activity of KCC2 is not particularly high. When the intracellular Cl^- concentration is increased (3), Cl^- ions bind to WNK and inactivate it. WNK is no longer able to activate SPAK and simultaneously, the inhibition of protein phosphatases is lost. Protein phosphatases thus are able to dephosphorylate KCC2 molecules (4) and the ion transport activity is increased.

5. CONCLUSIONS AND OPEN QUESTIONS

The KCC2 gene produces two isoforms, KCC2a and KCC2b, with different N-termini. Both isoforms are transcribed from different promoters, but the initial expression of the isoforms seems to be controlled by common mechanisms as both isoforms are largely neuron specific (Uvarov *et al*, 2007), and the pattern of KCC2a and KCC2b expression is similar in embryonic and neonatal mouse CNS (Uvarov *et al*, 2009) (Study II). The understanding of the mechanisms that control the initial neuron-specific expression of KCC2 isoforms still remain incomplete, but common enhancers or regulatory elements rather than individual promoter sequences might be important. For example, data on the involvement of NRSF in the neuron-specific expression of KCC2b are controversial (Karadsheh and Delpire, 2001, Uvarov *et al*, 2005, Yeo *et al*, 2009). Alterations of the chromatin state, for example DNA methylation, might also be an important component of regulation.

During the first postnatal weeks in mouse, KCC2b expression is strongly increased in cortical regions. The KCC2b promoter contains several binding sites for activity-dependent transcription factors that probably participate in the developmental upregulation of the KCC2b isoform, such as Egr4 (Uvarov *et al*, 2006) and USFs identified in study I of this thesis. In addition to Egr4 and USFs, several additional transcription factors are likely to participate in the transcriptional regulation of the KCC2b isoform, but have not been studied so far. The upstream signaling pathways involved in activation of transcription by USFs also remain to be identified.

Although KCC2 isoforms have an overlapping expression profile in the developing mouse CNS, KCC2a expression remains low in most cortical neurons in adult. The KCC2a promoter contains putative binding sites for general transcription factors that are not particularly enriched in neurons (Uvarov *et al*, 2007), and in line with this, KCC2a is not upregulated in neurons during postnatal development. In immunostainings performed in study II, KCC2a protein was detected in the basal forebrain, hypothalamus, and many areas of the brainstem and spinal cord, but not in most cortical regions in adult mouse brain.

Various data also indicate that KCC2a may exhibit a broader tissue distribution in vertebrates, in contrast to KCC2b, which seems to be predominantly CNS neuron specific. In one study KCC2a was the only variant detected in human lens epithelial cells (Lauf *et al*, 2012). In chicken heart, KCC2a was the predominant splice variant and was robustly expressed in cardiomyocytes (Antrobus *et al*, 2012). KCC2a (and a splice variant of KCC2a isoform lacking exon 24) was also the predominant isoform identified in pancreatic β-cells in mouse and human (Kursan *et al*, 2017). Thus, some role of KCC2a outside the CNS, for example in insulin secretion in pancreas, is also possible.

A downregulation of KCC2b at the mRNA and protein level is observed in various pathological conditions, and has been particularly studied in various forms of epilepsies. The mechanisms involved in the activity-dependent downregulation of KCC2b in pathological conditions have not yet been well characterized at the transcriptional level. When KCC2b is downregulated, NKCC1-mediated accumulation of intracellular chloride results in

depolarizing and even excitatory GABAergic events. GABA-mediated depolarizations might result in increased intracellular Ca^{2+} via voltage gated calcium channels, such as the L-type channels, or NMDARs. The increase in intracellular Ca^{2+} induces signaling events that may regulate the activity-dependent downregulation of KCC2b at the transcriptional level.

Increased neuronal activity under pathophysiological conditions may also lead to a downregulation of KCC2 transport function mediated by posttranslational modifications, such as changes in phosphorylation/dephosphorylation. For example, pathological-like patterns of neuronal activity have been demonstrated to initiate a rapid (30-60 min) withdrawal of KCC2 from the plasma membrane that leads to a reduction of KCC2-mediated ion-transport (Rivera *et al*, 2004). Levels of glutamate may increase within the brain during a seizure and mediate a NMDAR-dependent Ca^{2+} influx that activates protein phosphatase 1 and results in subsequent dephosphorylation of KCC2 S940 (Lee *et al*, 2011). Dephosphorylation was demonstrated to inhibit KCC2 activity and to decrease its membrane stability. Glutamate-mediated Ca^{2+} influx through activated NMDARs and S940 dephosphorylation also favored calpain cleavage of KCC2 C-terminal domain leading to KCC2 internalization and degradation (Puskarjov *et al*, 2012).

Mutation /polymorphism in the KCC2 gene has been identified in relationship to idiopathic epilepsies in humans (see Fig. 4). Mutations R952H and R1049C in KCC2 were found to be associated with idiopathic generalized epilepsy in humans, and both mutations decreased the level of KCC2 S940 phosphorylation and the plasmalemmal expression of KCC2 and acted in a dominant-negative manner (Kahle *et al*, 2014, Puskarjov *et al*, 2014). R952H substitution was also observed to cause a decreased dendritic spine density and altered spine morphology (Puskarjov *et al*, 2014). L311H, L426P and G551D mutations were identified as a cause of epilepsy of infancy with migrating focal seizures, and these mutations reduced the surface expression and glycosylation of KCC2 (Stodberg *et al*, 2015). Mutations M415V, A191V and S323P impaired the transport function of KCC2 but produced no changes in the surface expression was detected (Saitsu *et al*, 2016). A deletion associated with epilepsy, E50–Q93 in the N terminus caused by skipping of exon 3 also resulted in significant loss of KCC2 function (Saitsu *et al*, 2016).

Seizure-induced downregulation is likely to primarily involve the KCC2b isoform, as this isoform is widely expressed in the adult brain and known to be important for inhibition. This is also supported by observations in transgenic animals, as KCC2b knockout mice die a few weeks postnatally due to spontaneous seizures (Woo *et al*, 2002). The characterization of KCC2a-isoform deficient mice is still ongoing, but the mice appear to have a normal life span and do not present spontaneous seizures.

The KCC2a isoform could, however, be involved in the development of hyperexcitability and seizures in some brain regions. For example, in temporal lobe epilepsy, the most common type of acquired epilepsy in adults, downregulation of KCC2 mRNA and subsequent depolarizing GABAergic events has been observed in the hippocampal subiculum in human epileptic tissue (Palma *et al*, 2006, Huberfeld *et al*, 2007). When temporal lobe epilepsy was induced in rats by pilocarpine, a downregulation of KCC2 mRNA in many

parts of the hippocampal formation, including subicular neurons, was observed (Bragin *et al*, 2009, Barmashenko *et al*, 2011). Since the KCC2a isoform was exceptionally detected in the subiculum in immunohistochemistry stainings (study II), both KCC2a and KCC2b downregulation could potentially play a role in the initiation and maintenance of epileptic discharges in temporal lobe epilepsy.

In pyramidal neurons of the rat hippocampus, an activity-dependent increase in the surface expression of both KCC2 isoforms has been demonstrated during the first postnatal week (Khirug *et al*, 2010): A single kainite induced *in vivo* seizure episode during postnatal days 5–7 resulted in a fast increase in the Cl^- extrusion capacity of CA1 neurons, with a consequent hyperpolarizing shift of the reversal potential of GABAA-mediated currents. Biochemical data demonstrated an approximately twofold increase in the plasmalemmal pool of both KCC2a and KCC2b isoforms in the CA1 region. Thus, regulation of the surface expression of KCC2 isoforms may represent an intrinsic antiepileptogenic mechanism in neonates.

Following peripheral nerve injury, a reduction in KCC2 expression and activity has been observed downstream from the injured neurons, in lamina I of the superficial dorsal horn (Coull *et al*, 2003). Spinal cord neurons in lamina I receive inputs from pain-conducting C-fibers and project the pain signal to the brain, thus these neurons constitute one of the main spinal nociceptive output pathways. Cl^- accumulation and change in GABAA-mediated responses may cause normally inhibitory anionic synaptic currents to be excitatory. Antibodies that recognize both KCC2 isoforms were used in this study, and thus downregulation of both KCC2a and KCC2b expression/activity could be involved in the attenuated inhibitory action of GABA and glycine in central sensitization of the spinal cord.

In rats, spinal cord injury induced a downregulation of KCC2 in the spinal cord below the lesion, in the plasma membrane of motoneurons resulting in increased excitability of motoneurons and contributed to spasticity (Boulenguez *et al*, 2010). Also in this study, antibodies recognizing both KCC2 isoforms were used.

In study III, a similar ion transport activity of KCC2 isoforms was demonstrated in neuronal cells. The KCC2a isoform was able to mediate K-Cl cotransporter activity in overexpression experiments in KCC2-null neurons and in immature wild-type neurons. In line with this, KCC2a also appears to be functional in ion-transport *in vivo*, as KCC2b-deficient mice survive up to three weeks postnatally (Woo *et al*, 2002). The KCC2a N-terminal domain includes an SPAK-binding domain, and in study III we demonstrated that overexpressed SPAK in HEK293 cells bound to the N-terminus of KCC2a, but not that of KCC2b. Different regulation of the transport activity of the isoforms by SPAK was also observed in HEK293 cells in study III. Thus, the isoform-specific N-terminal parts could have important regulatory properties.

Phosphorylation is known to play a crucial role in the regulation of the intrinsic transport function of KCC2. For example, S940 in the C-terminal domain of KCC2 is a site for protein kinase C (PKC), and phosphorylation at S940 has been shown to increase the transport rate of KCC2 and stability at the plasma membrane (Lee *et al*, 2007, Lee *et al*, 2011). In contrast,

KCC2 activity at the plasma membrane can be inhibited by WNK-SPAK kinases at T906/T1007 (Kahle *et al*, 2016). Studies have also shown that KCC2 is more highly phosphorylated at T906/T1007 by WNK-SPAK in immature neurons compared to mature neurons (Friedel *et al*, 2015). Thus, regulation by WNK/SPAK kinases and dephosphorylation at T906/T1006 seems to be important in postnatal activation of KCC2 function.

Since the WNK-SPAK pathway is important for the GABA shift in development (Friedel *et al*, 2015), a major open question is whether the difference in T906/T1007 phosphorylation in development is KCC2 isoform-specific and how the phosphorylation (and thus activity) of the KCC2 isoforms by WNK-SPAK pathway differs in neurons during postnatal development. *Thus, the phosphorylation profile of KCC2 isoforms should be further investigated in primary neurons during development.*

The regulation of KCC2 activity via the WNK-SPAK pathway also appears to be important in neuropathic pain: Mutation in a WNK1 isoform that is expressed in the nervous system, and particularly in the dorsal horn of the spinal cord, results in hereditary sensory and autonomic neuropathy type IIA (HSANII) in humans. A knockout mouse specifically lacking this WNK1 isoform had less phosphorylation of KCC2 at T906 and T1007 in the spinal cord and an increase in KCC2 activity, and the mice were less susceptible to hypersensitivity after peripheral nerve injury (Kahle *et al*, 2016). KCC2 T906 and T1007 phosphorylation was decreased and EGABA was restored to more negative values in lamina II neurons from mice with nerve injury by slice incubation with STOCK1S-50699 (an inhibitor of the WNK-SPAK pathway), resulting in reduced cold allodynia and mechanical hyperalgesia (Kahle *et al*, 2016).

Since chronic neuropathic pain is often resistant to treatment, a novel potential analgesic strategy in neuropathic pain would be to restore GABA-mediated inhibition by enhancing KCC2 activity in dorsal horn neurons (Gagnon *et al*, 2013). Pharmacologically antagonizing the WNK/SPAK pathway would promote combined NKCC1 inhibition and KCC2 stimulation, also yielding a potent strategy to enhance cellular Cl^- extrusion and the GABA inhibition in neuropathic pain after nerve injury (Kahle *et al*, 2013). Since both KCC2 isoform are expressed in the spinal cord, KCC2a could also contribute to pain hypersensitivity after nerve injury, particularly since regulation by WNK-SPAK pathway seems to be critically involved. *An interesting question is also the difference between the phosphoregulation of KCC2a vs. KCC2b by the WNK-SPAK pathway after trauma.*

Novel specific and potent pharmacological compounds that modulate KCC2 activity have been identified in high-throughput screens. *To further study the differences between the two isoforms, the isoform-specificity of these compounds could be tested in HEK293 cells overexpressing KCC2 isoforms.* For example, the KCC2 selective antagonist VU0463271 has been discovered that efficiently inhibited KCC2b overexpressed in HEK293 cells in Rb uptake assay (Delpire *et al*, 2009, Delpire *et al*, 2012). However, VU0463271 was found to have poor pharmacokinetic properties, because of rapid metabolism (Delpire *et al*, 2012). *In vivo* infusion of the inhibitor into hippocampus of adult WT mice resulted in hyperexcitability and epileptiform discharges in the hippocampal slices (Sivakumaran *et al*, 2015). Experiments with VU0463271 also

demonstrated that reduced KCC2 transport increased the duration of seizure-like events in acute mouse brain slices in the 0-Mg^{2+} model (Kelley *et al*, 2016).

KCC2 activators would potentially be of use therapeutically, in the treatment of epilepsy, neuropathic pain and other disorders. So far, a KCC2-selective enhancer, CLP257, has been discovered that significantly increased the rate of Cl^- accumulation and alleviated hypersensitivity in a rat model of neuropathic pain (Gagnon *et al*, 2013). The CLP257 compound appears to modulate plasmalemmal KCC2 protein turnover, most likely by inhibiting internalization of KCC2. *Although the mechanism has not been fully resolved, it would also be useful to determine possible isoform-specific effects of this this compound.*

By confocal immunofluorescence microscopy, a partially different distribution of KCC2a and KCC2b at the subcellular level was observed in the dendritic compartments of mature neurons in mouse brain sections (Study II). KCC2a was seen in discrete puncta particularly in distal dendrites which did not always co-localize with KCC2b. Distinct isoform-specific compartmentalization might suggest unique functions of the isoforms in neurons. It is possible that the unique N-terminal regions of the isoforms dictate their subcellular localization by interacting with different trafficking elements. KCC2 isoforms might accumulate in different subdomains at the plasma membrane, for example in lipid rafts. Alternatively, KCC2a specific puncta may represent accumulation in intracellular compartments in distal dendrites. *A future task would be to address the different subcellular localization of KCC2 isoforms within distal dendrites using electron microscopy.*

Differential surface expression of KCC2 isoforms was also observed in immunostainings. KCC2b signal was present at the plasma membrane throughout the somatodendritic compartments, while KCC2a was not detected at the neuronal plasma membrane in most brain regions in mouse sections (Study II and III). In a few brain regions (most clearly seen in the subiculum in study III) KCC2a immunoreactivity was detected at the cell surface. In primary rat cortical or mouse hippocampal neuronal cultures, KCC2b but not KCC2a immunoreactivity was observed nearby the plasma membrane surface of soma and proximal dendrites (Study III). However, biotinylation experiments indicated that both isoforms were expressed to a similar extent at the neuronal plasma membrane, suggesting that the KCC2a N-terminus at the cell surface may be masked. Masking could suggest that KCC2a is also at the membrane *in vivo* even if we do not detect this in most brain regions (indeed immunohistochemistry data from KCC2b knockout mice in study III support the masking *in vivo*).

Protein interactions, oligomerization, phosphorylation or other modifications could mask the KCC2a N-terminal epitope at the neuronal surface. Since a differential interaction of the KCC2 isoforms with SPAK was demonstrated in HEK293 cells, SPAK could be one possible candidate responsible for masking of the KCC2a N-terminal epitope. On the other hand, the N-terminal epitope could also be masked by not yet identified protein interactions. *It would be useful in mass spectrometry experiments to determine the KCC2a N-terminal binding partners in neuronal cultures, as well as to directly confirm the binding of SPAK.* Identifying KCC2a –specific interacting

partners should aid in determining the mechanism of masking, and also help to clarify the specific role of the KCC2a isoform in neurons.

KCC2 has been shown to engage in a variety of protein-protein interactions. Recently, a global KCC2 interactome study was performed by affinity purification of KCC2 from mouse whole brain membrane fractions (Mahadevan *et al*, 2017). High-resolution mass spectrometry analysis revealed 150 previously unknown native-KCC2 interactors in mouse brain. Some of the interacting partners were enriched exclusively from mature brain and others only from developing brain (P5). While isoform specific-interactions were not determined in this study, it was observed that different detergents extracted KCC2 isoforms differentially together with largely different subsets of proteins, indicating that detergent stabilities of KCC2 isoforms and their associated protein complexes are different.

Many of the new interacting partners that were identified in the global KCC2 interactome study were exclusively enriched at excitatory synapses and particularly many were involved in receptor trafficking (Mahadevan *et al*, 2017). The most abundant KCC2 interactor in this study, PACSIN1, is a neuronal endocytic adapter protein that regulates the surface expression of distinct glutamate and glycine receptors.

Various ion-transport-independent roles of KCC2 in spinogenesis has been demonstrated through structural interactions with components of the actin cytoskeleton. KCC2-deficient neurons demonstrated an aberrant maturation of dendritic spines and a decrease in the number of functional synapses (Li *et al*, 2007, Gauvain *et al*, 2011). Dendritic spines of KCC2-deficient neurons exhibited increased stability of actin filaments and reduced spine motility (Llano *et al*, 2015). In mature hippocampal cultures, immunostaining with KCC2 pan-antibodies demonstrated that KCC2 is expressed at higher levels in mushroom spines (with active synapses) and at lower levels in filopodia (Llano *et al*, 2015).

The structural interactions important for morphology and function of spines seem to involve the common C-terminal domain of KCC2 (Chevy *et al*, 2015, Li *et al*, 2007, Llano *et al*, 2015), and the interactions could thus engage both isoforms. However, KCC2b is widely expressed in cortical regions and its upregulation in development temporally parallels spinogenesis and excitatory synaptic maturation. KCC2a is for the most part not expressed in cortical regions, and is thus supposedly not important in spines. However, KCC2 isoforms could also be involved in actin regulation in other cellular compartments in addition to spines, although this aspect has not yet been studied. A specific structural role of KCC2a is also possible, perhaps via sites of interaction in the unique N-terminal part.

In conclusion, the isoform-specific functional role of KCC2a remains to be studied. Different underlying regulatory mechanisms and post-translational modifications are presumably important in defining the unique functions of isoforms in neurons. Further work is required to understand in detail how the WNK-SPAK pathway controls phosphorylation and activity of the KCC2 isoforms in hetero- as well as homodimers. In addition, a specific role of the KCC2a isoform outside the CNS is also possible, and remains to be studied in future

experiments. Ongoing and planned studies in KCC2a knock out mice are seeking to answer the question of the KCC2a role *in vivo*.

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